Claim 34, line 1, delete "or 32" after "claim 31".

REMARKS

Applicant and his representatives gratefully acknowledge the helpful assistance which the Examiner provided in their November 21, 1996 interview in this application. Claims 31, 33 and 34 are pending in this application. A copy of the pending claims, as amended herein, is attached for the Examiner's convenience.

Applicant has amended claim 31 to recite more clearly what applicant considers to be his invention. First, applicant amended claim 31 to identify more clearly the recipient of the claimed method as "a patient in need of such treatment". Second, amended claim 31 recites a "recombinant polypeptide produced by a non-human host". These amendments are supported by the specification as filed. None adds new matter.

The Examiner has noted that informal drawings have been filed. Formal drawings will be filed when the application is allowed.

The Examiner's rejections are addressed below.

THE REJECTIONS UNDER 35 U.S.C. § 103

The Taniguchi/Roberts/Borden Combination

The Examiner has rejected claims 31-34 under 35 U.S.C. § 103 as "unpatentable" over Taniguchi et al., Gene,

10, pp. 11-15 (1980) ("Taniguchi") in view of Roberts, Proc. Natl. Acad. Sci. USA, 76, pp. 5596-600 (1979) ("Roberts") and further in view of Borden et al., Annals of Internal Med., 91, pp. 472-79 (1979) ("Borden"). The Examiner contends that it would have been obvious for one of skill in the art at the time the invention was made to express the human IFN-ß1 gene of Taniguchi using methods disclosed in Roberts to produce large amounts of IFN-ß1 for anti-tumor therapy as suggested by Borden. Applicant traverses this rejection based on the specific facts of this case as well as the law, as described in detail below.

The Examiner's rejection requires first, that the combination of Taniguchi and Roberts be proper, and second, that the combination disclose or suggest the recombinant IFN-ß polypeptide recited in applicant's amended claims. Neither requirement is met. Hence, the rejection fails.

1. The Combination Of Taniguchi And Roberts Is Improper

For reasons previously made of record in this case, there is no basis for the Examiner's combination of Taniguchi and Roberts. Neither document specifically teaches or suggests the combination: Taniguchi says nothing about IFN-β expression and Roberts says nothing about expressing in bacteria a cellular eukaryotic protein

having biological activity, much less applicant's recombinant IFN-ß having antiviral activity.

The Examiner points to Roberts, p. 5600, last paragraph, to support this combination of references. According to the Examiner, this paragraph suggests that the Roberts approach, successful for expressing SV40 t antigen, would be successful for expressing other eukaryotic genes in bacteria.

However, all evidence is to the contrary -- the ordinarily skilled artisan would not have made the Taniguchi/Roberts combination, given the information that was available at the effective filing date of this application, with any expectation of success in being able to express in bacteria IFN-β1 polypeptides having antiviral activity.

2. **There Was No Reasonable Expectation Of Success**

There was no reasonable expectation of success in producing recombinant IFN-ß in 1980 before applicant achieved it for the first time. The Roberts approach could not and did not address the specific problems inherent in the expression of IFN-ß. For this reason, the ordinarily skilled artisan would not have selected the Roberts approach in attempts to express IFN-ß.

Applicant enclosed with his July 16, 1996 Amendment copies of the Declaration of Richard L. Cate and

the Supplemental Declaration of Richard L. Cate, which describe the problems inherent in recombinantly expressing IFN-ß. Cate Supp. Decl. ¶ 26.

Dr. Cate's declarations detail the expected problems in the recombinant production of IFN-ß and explain why these problems did not provide the ordinarily skilled artisan with a reasonable expectation of success even with Roberts in hand. Cate Decl. ¶¶ 9-17, 22-35, 39-44, 46, 52, 61, 65; Cate Supp. Decl. ¶¶ 3-4. To emphasize the point, Dr. Cate expressly stated that expression of recombinant IFN-ß was "unpredictable." Cate Decl. ¶ 11; Cate Supp. Decl. ¶ 4. The expected problems described by Dr. Cate include the marked differences between SV40 t antigen and IFN-ß. Because of these differences, there is simply no basis for selecting the Roberts approach over any other possible approach known in 1980. Cate Supp. Decl., ¶ 26.

Further, Roberts does not solve any of the specific or general problems confronting one of skill in the art trying to express IFN-ß.

Taniguchi (as well as other prior art) identified the unique properties of IFN-ß. Based on these properties, the ordinarily skilled artisan would have expected problems in the recombinant production of IFN-ß. These included problems with proteolytic degradation, bioinactivity, toxicity, and insolubility. Cate Supp. Decl. ¶¶ 7-8. Specific problems due to IFN-ß's three cysteine (Cys)

residues and extreme hydrophobicity were also expected. Cate Supp. Decl. ¶¶ 9-23. There was no reasonable expectation of success in overcoming those problems. Cate Supp. Decl. ¶¶ 23, 27-28. See also decision of the Board of Appeals and Patent Interferences, Goeddel v. Weissmann, Interference 101,601, Paper No. 265 (December 15, 1995) ("Goeddel v. Weissmann Decision") (copy enclosed with applicant's July 16, 1996 response) at 24-26.

Further, in 1980 the ordinarily skilled artisan could not have ruled out the possibility that IFN-ß underwent post-translational processing during expression and secretion. This would have been a concern because of the discrepancy between the molecular weight of IFN-ß as measured with the native glycosylated protein and that predicted from the sequence reported in Taniguchi. Cate Decl. ¶ 22. See also Fantes, p. 177 (Cate Supp. Decl., Exhibit 20).

In 1980, the ordinarily skilled artisan could not have predicted that such post-translational processing would occur correctly (or at all) in a heterologous host. For this reason too, then, there was no reasonable expectation of success in attempts to produce recombinant IFN-ß. This is supported by the Board in Ex parte Goeddel, Appeal No. 94-2099 (August 31, 1994) (copy enclosed with applicant's July 16, 1996 response), which stated that one skilled in the art in 1980 would not have been able to

predict whether non-glycosylated IFN-β (one class of applicant's non-human recombinant interferons) would be biologically active. As a consequence, they also could not predict that the antiviral interferon polypeptides recited in claims 31-34 could be used in those claimed methods for treatment.

Perhaps, the best evidence that on June 6, 1980, one of ordinary skill in the art would not have had a reasonable expectation of success using Roberts to express IFN-ß is what the skilled workers actually did at the time. None of the workers in 1980 used the Roberts approach in their attempts to express IFN-ß -- they chose not to make the combination that the Examiner is making in hindsight some 15 years later. Cate Decl. ¶ 62.

Dr. Roberts himself (who best knew the Roberts approach) and Dr. Taniguchi (who best knew the problems inherent in the IFN-β sequence) did not use the Roberts approach when they expressed recombinant IFN-ß -- after applicant. Instead, they used a new method, referred to in Guarente, published after applicant's June 6, 1980 priority date. Guarente expressly states that they developed a new method for recombinant expression because identification of expressing transformants using the Roberts approach "may be laborious or impossible." Guarente, p. 544; Cate Decl. ¶ 62.

This contemporaneous attitude towards the "Roberts approach" is powerful evidence of non-obviousness. See Interconnect Planning Corp. v. Feil, 774 F.2d 1132, 1143 (Fed.Cir. 1985) (stating "[a] retrospective view of the invention is best gleaned from those who were there at the time").

Dr. Goeddel also subsequently expressed IFN-ß. He too did not use the Roberts approach. Cate Decl. ¶ 62.

Furthermore, Dr. Taniguchi and Dr. Roberts themselves thought that the expression of IFN-ß was patentable over their own previous work. They filed a patent application claiming that subject matter. See EP-A 0 042 246; Cate Decl. ¶ 63. So did Dr. Goeddel. See EP-A 0 048 970; Cate Decl. ¶ 63. And, the Board has held Goeddel's expression of a subgenus of IFN-β -- microbially produced, mature, non-glycosylated IFN-β -- to be patentable in September 1980 -- three months after applicant expressed IFN-β for the first time. See United States patent 5,460,811 ("the '811 patent") (cited in the Supplemental Information Disclosure Statement filed July 16, 1996), which was allowed over both Roberts and Taniguchi, cited here. See also the '811 patent, p. 1, column 2, line 26 and p. 2, column 1, lines 16-17.

All of the foregoing demonstrates that there was no reasonable expectation of success in attempts to produce

recombinant IFN-ß at applicant's June 6, 1980 priority date.

3. The Claims Are Patentable Over the Taniguchi/Roberts Combination

Even if the Taniguchi/Roberts combination was proper -- which it is not -- neither Taniguchi nor Roberts, alone or in combination, disclose or suggest the recombinant polypeptide displaying antiviral activity in the treatment methods recited in applicant's claims.

The IFN-β gene of Taniguchi was a "copy DNA" (cDNA) on a bacterial plasmid (pBR322) unlinked to any plasmid expression control sequences. Because cDNA is enzymatically copied messenger RNA (mRNA), which starts downstream from the major expression control sequences of the gene (e.g., promoter and enhancer elements), the human IFN-β cDNA of Taniguchi was also not linked to any endogenous expression control sequences.

The Examiner does not disagree that expression or even capability for expression was missing from Taniguchi. Instead, the Examiner contends that it would have been obvious for the skilled practitioner to make a recombinant DNA molecule capable of expressing Taniguchi's IFN-β cDNA by inserting that cDNA into the bacterial expression vector of Roberts. This contention, however, is unwarranted for several reasons.

First, Roberts described a bacterial plasmid in which the simian virus SV40 small tumor ("t") antigen gene was fused precisely to transcription and translation control sequences of the E. coli lac operon to create a "hybrid ribosome-binding site." Roberts, Abstract, lines 7-11. (This differs from a vector in which a heterologous promoter is placed upstream from a gene which retains its natural translation initiation sequences). Bacteria transformed with such constructs were screened for SV40 expression products using antibody-containing serum from animals harboring SV40-induced tumors. SV40-related immunoreactive polypeptides were then characterized.

Roberts concluded that in order to express the t antigen from such fusion plasmids, a fairly precise juxtaposition between the translation initiation codon (ATG) of the SV40 t gene and the lac operon Shine-Delgarno (SD) sequences (i.e., the ribosome binding site) was required:

> "synthesis is barely detectable if the distance between the *lac* SD sequence and the ATG of t is large (17 base pairs).... support[ing] the notion that formation of a hybrid ribosome-binding site bearing appropriately positioned SD and ATG sequences is essential to translation of t."

Roberts, p. 5600, Discussion, first paragraph (emphasis added).

Significantly (and a point that the Examiner ignores), Roberts did not teach or suggest that this

specific vector and distance constraints would be successful for expressing any other eukaryotic gene in bacteria. In the words of Roberts, their experiment provided a "rational approach [not a solution] to the problem of obtaining expression of eukaryotic genes in bacteria." Roberts, p. 5600, last sentence.

Nor did Roberts teach or suggest that any other DNA sequence -- prokaryotic or eukaryotic -- could be fused to the lac operon of E. coli with the same distance constraints shown to be required to achieve SV40 t gene expression (the lac SD sequence and ATG being separated by no more than 17 base pairs), to express successfully a gene product encoded by that DNA sequence in bacteria. In fact, a slightly earlier publication by the authors of Roberts* ("Roberts II")(enclosed as **Exhibit A**) suggested just the opposite:

(1) that juxtaposition between the lac SD sequence and the heterologous ATG codon was not in itself sufficient to guarantee successful expression of a polypeptide; and

(2) that 16 or more nucleotides between the lac SD sequence and the heterologous ATG codon were required for efficient translation of λ cro protein.

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* Roberts et al., "A general method for maximizing the expression of a cloned gene," Proc. Natl. Acad. Sci. U.S.A., 76, pp. 760-64 (1979).

In Roberts II, the E. coli lac promoter and Shine-Delgarno (SD) sequences were fused at increasing distances from the ATG codon of the phage λ cro gene -- a prokaryotic gene. Six out of nine plasmids expressed the heterologous prokaryotic protein (cro) but with "striking differences among them ... [and] with no explanation for these differences." Roberts II, p. 763, paragraph 2; see also Figure 3.

The enormous differences in the amount of cro protein produced by these plasmids did not correlate simply with the distance separating the lac SD sequence from the cro ATG codon. One plasmid which expressed very little cro protein differed from the highest expressing fusion plasmid by only 3 base pairs (bpr) in the distance between the lac SD sequence and cro ATG codon. See Figure 3, p. 763 (38 bpr (pTR199) and 41 base pairs (pTR213), respectively.

Thus, taken together, Roberts and Roberts II do not teach that the SV40 t gene of Roberts could simply be replaced with any other eukaryotic gene sequence and be successfully expressed in E. coli. These articles suggest the opposite. They suggest that every gene will likely behave differently when fused to upstream sequences of the E. coli lac operon. And, they suggest that more than just a Shine-Delgarno sequence juxtaposed to an ATG codon is required for successful translation of a heterologous protein in E. coli.

Also, by early 1980 it was apparent to several other groups, including applicant's, that secondary structures formed between sequences surrounding and encompassing the 5' untranslated region and the translation initiation codon were capable of significantly affecting the efficiency and fidelity of translation initiation. Reviewed in Steitz, J.A., "Genetic Signals and Nucleotide Sequences in messenger RNA," in Goldberg, R.F. (Ed.), Biological Regulation and Development: I. Gene Expression, Plenum, New York, 1979, pp. 349-389 (copy enclosed as **Exhibit B**). See especially Sections 2.6, "mRNA Structure and Initiation," pp. 363-367; and Section 2.10, "Perspectives and Problems," paragraph 1.

That secondary structure considerations could be important for heterologous gene expression was noted in a publication from early 1980 co-authored by applicant*:

> Finally, it may be noted that the effects of mRNA secondary structure on translation as proposed in the present paper are of particular relevance for expression of a cloned foreign gene. Not only a proper start of transcription and a ribosome interaction site have to be provided, but furthermore the accessibility of the initiation sequence including the AUG (or GUG) in the secondary structure must be considered....

Page 11, last paragraph.

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* Iserentant and Fiers, "Secondary Structure of mRNA and Efficiency of Translation Initiation," Gene, 9, pp. 1-12 (1980) (copy attached as **Exhibit C**).

For all the above reasons, at the time this invention was made, it could not have been obvious to one of skill in the art that any gene could be cloned into a Roberts vector with the distance constraints taught for the SV40 t antigen to produce a different heterologous polypeptide in E. coli. The art taught that each gene would behave differently in that system.

In addition, Roberts failed to show that SV40 t polypeptides expressed in bacteria from lac fusion constructs possessed any biological activity. The t polypeptides were detected based only on their ability to bind to SV40-specific antibodies. Thus Roberts failed to teach or suggest that their method could be used to express and isolate a biologically active SV40 t antigen, let alone a biologically active human IFN-β (or any other eukaryotic gene product) in bacteria or in any other host. Yet, all of applicant's claims recite a recombinant polypeptide "displaying antiviral activity" and thus require that the IFN-β polypeptide possess biological activity. This failure of Roberts is not solved by Taniguchi, which, as the Examiner has conceded, did not teach an IFN-β expression plasmid or expression products.

The Examiner's rejection is also totally inconsistent with two recent decisions of the Board of Appeals and Patent Interferences: (1) Goeddel v. Weissmann,

supra; and (2) Ex parte Goeddel, supra, for the reasons set forth in applicant's July 16, 1996 Response.

Those decisions by two different panels: (1) APJs R. Smith, Downey and W. Smith (Goeddel v. Weissmann); and (2) APJs Winters, W. Smith and Gron (Ex parte Goeddel); specifically address the same issue that underlies the Examiner's Section 103 rejection: with the DNA sequence for an interferon in hand, would it have been obvious to the skilled person to express that DNA and produce biologically active interferon in April 1980 and in September 1980 (two months before and three months after applicant's June 6, 1980 latest priority date here). Resoundingly, both Boards said NO. Fundamental fairness requires a consistent application of that law here.

For all the reasons discussed above, the combination of Taniguchi and Roberts is both an improper one and one which provided no expectation of success to those of skill in the art at the effective filing date of this invention. In addition, even upon making this improper combination, Taniguchi and Roberts alone and in combination fail to disclose or suggest applicant's claimed invention.

The addition of Borden and the prior art knowledge that native human IFN-β has antitumor and other therapeutic activities fails to remedy the defects in the primary Taniguchi/Roberts combination. Because applicant's

claimed recombinant polypeptides are novel and non-obvious over the prior art, their use in a method for treating various diseases by prior art methods must also be novel and non-obvious.

For all of these reasons, the applicant's amended claims are not obvious over the cited art. Accordingly, applicant requests that the Examiner withdraw the Section 103 rejections.

CONCLUSION

For all of the above reasons, reconsideration and allowance of the pending claims is requested.

Respectfully submitted,

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I Hereby Certify that this Correspondence is being Deposited with the U.S. Postal Service as First Class Mail in an Envelope Addressed to: ASSISTANT COMMISSIONER FOR PATENTS WASHINGTON D.C. 20231, on April 15, 1997

Thomas Quinones
Name of Person Signing

Signature of Person Signing

Roberts

Proc. Natl. Acad. Sci. USA
Vol. 76, No. 2, pp. 760–764, February 1979
Biochemistry

A general method for maximizing the expression of a cloned gene

(exonuclease III/nuclease S1/*lac* promoter/deletion formation/*cro* protein)

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Communicated by Paul Doty, December 6, 1978

ABSTRACT **We present a method, utilizing a combination of restriction endonuclease cleavage and digestion with *Escherichia coli* exonuclease III and *Aspergillus orizae* nuclease S1, that allows us to position a restriction fragment bearing the promoter of the *lacZ* gene of *E. coli* at virtually any distance in front of any cloned gene. In particular, we have used this method to examine the effect on protein production of gene–promoter separation for the *cro* gene of phage λ and to produce plasmids that, upon transformation into appropriate *E. coli* hosts, direct the synthesis of up to 190,000 *cro* protein monomers per cell.**

Previous reports from this laboratory have described the use of a DNA fragment generated by restriction endonuclease cleavage as a "portable promoter." This fragment bears the promoter of the *lac* operon of *Escherichia coli* and is capable of directing efficient transcription *in vivo* of genes to which it has been fused *in vitro*. The fusions are carried on plasmids derived from pMB9 (1, 2). In the case of the *cI* gene of phage λ, the amount of *cI* protein (λ repressor) produced by a strain bearing a given *cI–lac* promoter fusion was a sensitive function of the gene–promoter separation. Strains bearing one of these plasmids, pKB280, direct the synthesis of over 30,000 monomers of *cI* protein per transformed cell, or roughly 150-fold more repressor than is found in a typical λ lysogen. In pKB280 the DNA fragment containing the *lac* promoter was abutted to the end of the *cI* gene so that the ribosome binding site (see *Discussion*) of the fusion was a hybrid of *lac* and λ sequences. Fusions which placed the *lac* promoter at a considerably greater distance from the *cI* gene produced 1/5th to 1/10th the amount of *cI* protein. In these experiments, however, the actual number of fusions explored was limited by the availability of convenient restriction cuts in close proximity to the 5′ terminus of *cI*.

In this communication, we describe a method that, in principle, will allow the same *lac* promoter fragment to be placed at virtually any distance in front of a gene. The promoter fragment does not encode a translational start. It does, however, encode a sequence required for binding the message to the ribosome (see *Discussion*). Therefore, our gene–promoter fusions will produce a native protein rather than a fusion protein carrying foreign amino-terminal amino acids (3, 4). In particular, we present details of the construction of a series of *lac* promoter fusions to the *cro* gene of λ designed to examine systematically the effect on protein production of gene–promoter separation, and to produce a strain that synthesizes large amounts of *cro* protein. The best of these strains directs the synthesis of 190,000 monomers of *cro* protein per cell.

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EXPERIMENTAL PLAN

Our basic approach is to clone a gene in a plasmid such that a unique restriction endonuclease cleavage site is located near (within approximately 100 base pairs of) the 5′ end of the gene. We then open the plasmid at that site and excise varying amounts of DNA with exonuclease III and the single-strand-specific endonuclease S1. We then insert a small DNA fragment bearing the promoter of the *lac* operon of *E. coli* and close the plasmid. This produces a set of plasmids bearing the promoter separated by varying distances from the gene. The specific procedure we used to construct the various *lac* promoter–*cro* gene fusions is diagrammed in Fig. 1. As indicated in the next paragraph, many of these steps might easily be modified to accommodate differing configurations of restriction enzyme cuts surrounding different genes. The essential features of the experiment of Figure 1 are as follows: (*i*) the *cro* gene was cloned: a DNA restriction fragment 550 base pairs long and bearing the *cro* gene was sheared to roughly 260 base pairs. Chemically synthesized *Bam* linkers (5) were ligated to the ends of this shorter fragment, and the resulting fragment was cloned into the *Bam* site of pBR322 (6) yielding pTR116. (*ii*) The *Bam* cut at the carboxy end of the *cro* gene in pTR116 was removed: the plasmid was partially digested with *Bam*; the resulting *Bam* sticky ends were rendered flush with *E. coli* DNA polymerase I and the four deoxynucleotide triphosphates (1). This left a single *Bam* cut in the resulting plasmid (pTR151) 54 bases from the amino terminus of the *cro* gene. (*iii*) Some or all of the 54 base pairs of DNA between the *Bam* cut and the ATG signaling the start-point of translation were removed: pTR151 was cut with *Bam* and resected for various times with *E. coli* exonuclease III with subsequent treatment with *Aspergillus orizae* nuclease S1 to remove single-stranded tails. (*iv*) The plasmid was cut at the unique *R* I site some 375 base pairs upstream from the *Bam* site, and an *R* I–*Alu* restriction fragment containing the *lac* promoter was inserted into the plasmid backbone. After transformation into *E. coli*, the resulting plasmids were characterized as described below.

In theory, the *cro* gene in the above construction could have been any gene, and the *Bam* site could have been any unique restriction site. If the *Bam* site had been located inconveniently far from the beginning of the gene, it could have been moved closer by opening the plasmid with *Bam*, digesting with *Exo* III and S1, and then religating the resulting plasmid in the presence of an excess of *Bam* linkers. The *R* I site utilized in the *cro* construction can also be substituted for by several other restriction sites on pBR322 (e.g., *Pst*, *Bam*, *Hin* III, or *Sal* I). We wish to emphasize the convenience of the construction used: the most difficult step, the original cloning of the gene, is done once and then left unchanged. The only inserted fragment, the *lac* fragment, bears a *lac* operator. Cells bearing plasmids

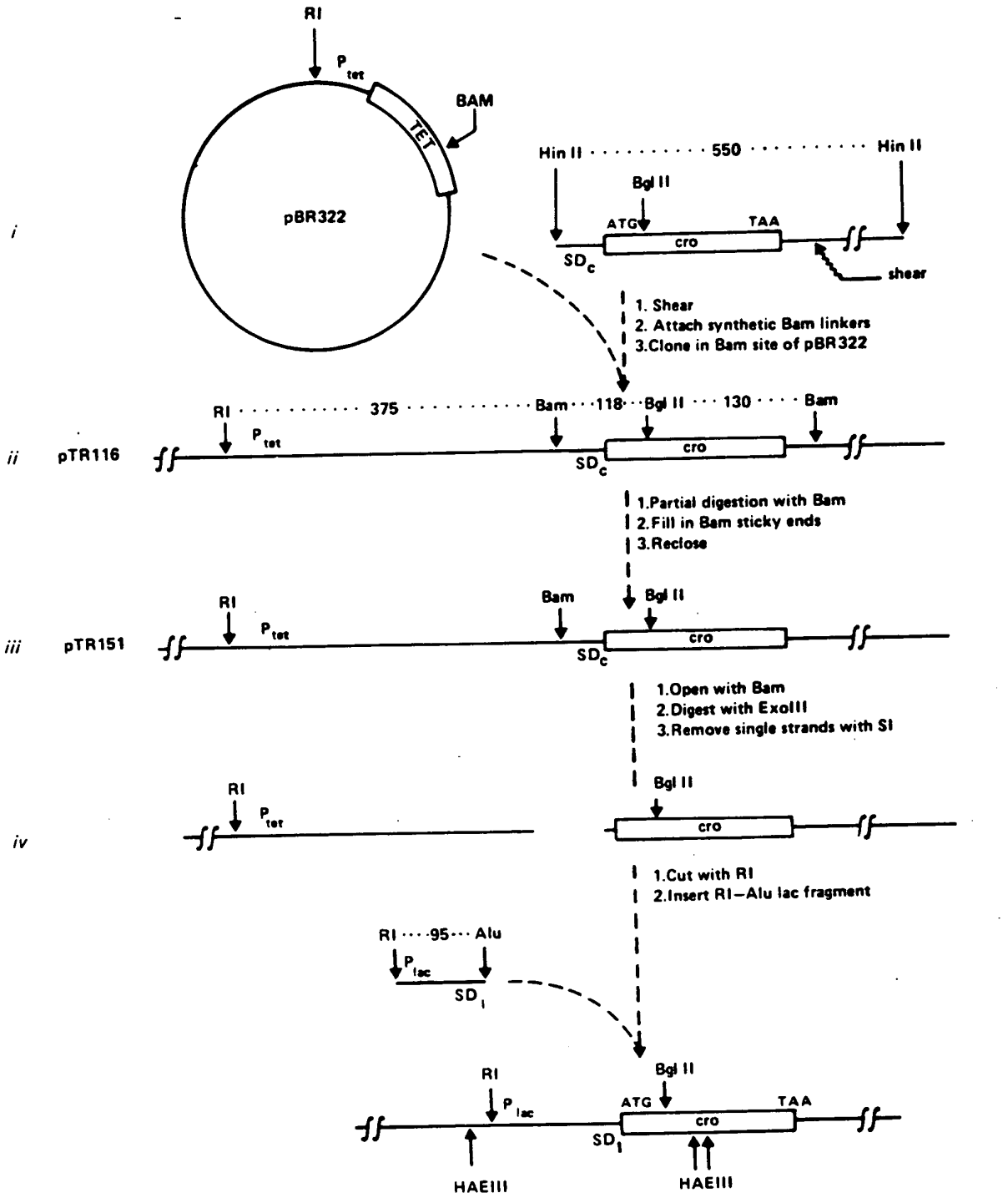

FIG. 1. Schematic representation of the methods of plasmid construction. The approximate locations of several restriction endonuclease cleavage sites are shown for the plasmid pBR322, for a DNA fragment bearing the *cro* gene of phage λ, and for a DNA fragment bearing the promoter of the *lacZ* gene [see Backman and Ptashne (2) for the source of this fragment]. The location of the *tet* and *lacZ* gene promoters are indicated, as are the extent of the *tet* and *cro* genes. SD_c and SD_1 indicate the Shine–Dalgarno sequences of the *cro* and *lacZ* genes, respectively. AUG and UAA are the start and stop signals for translation of the *cro* protein. Distances are indicated in base pairs. Steps: (*i*) The fragment bearing the *cro* gene was shortened by shearing to remove certain λ control elements near the 3′ end of the gene, and the smaller fragment was inserted into the *Bam* site in pBR322 by using *Bam* linkers. (*ii*) The *Bam* site near the carboxy terminus of the *cro* gene was eliminated. (*iii*) The plasmid was opened at the *Bam* site and varying amounts of DNA were removed by *Exo* III and S1. (*iv*) The partially resected plasmid was cut at the *R* I site, and the *lac* promoter (bearing the UV5 mutation rendering it independent of catabolite activator) was inserted by "sticky end" ligation at its *R* I end and by "blunt end" ligation to the resected plasmid DNA at its *Alu* end. The efficiency of steps *iii* and *iv* are fairly high—about 200–400 plasmids result from 1 μg of pTR151 input.

carrying the operator are easily recognized on indicator plates. Plasmids bearing the *lac* operator cause colonies of cells to turn blue on the appropriate indicator plate, because multiple copies of the operator titrate the *lac* repressor resulting in synthesis of β-galactosidase (see ref. 1).

MATERIALS AND METHODS

Strains. *E. coli* strain 294 (*Endo I*, $r_k^- m_k^+ B_1^- pro^-$) was used as the host for all plasmids. Cells were grown in TB medium supplemented with 0.1% yeast extract (7). Phage strains λKH54 (8), λ4*V'S* (V_2 V_{305} V_{3c} V_{S236}) (9), and λc411 [selected from 4*V'S* by growth on strains producing large amounts of *cro* (J. Eliason, unpublished results)] were used for cross-streaking.

Enzymes. T4 ligase and *E. coli* DNA polymerase I were used as described in Backman *et al.* (1). *Bgl* II restrictions were carried out in 6.6 mM Tris·HCl, pH 7.4/6.6 mM $MgCl_2$/6.6 mM 2-mercaptoethanol. *Bam*, *R I*, and *Hae* III digestions were carried out in the same buffer plus 60 mM NaCl. Exonuclease III (10) digestions were carried out at 22.5°C in the same buffer used for *Bam* digestion in 25–50-μl reaction mixtures at a DNA concentration of 100 μg/ml by using eight units of exonuclease III per μg of DNA. Under these conditions *Exo* III digests at a rate of 8–10 base pairs per min per end depending on the batch of enzyme. Exonuclease III digestion was halted by addition of an equal volume of two times concentrated S1 buffer: 100 mM NaOAc-HOAC, pH 4.0/300 mM NaCl/12 mM $ZnSO_4$. S1 nuclease was added to a concentration of 75 units per 50 μl and allowed to digest for 2 hr at 18°C (11). The reaction was stopped by phenol extraction, and the DNA was purified over a G-50 fine Sephadex column before further reaction.

Plasmid Constructions. *pTR116.* The 550-base-pair *Hin* II restriction fragment (5 μg) bearing the *cro* gene was dialyzed against 100 mM NaOAc, pH 8.0/1 mM EDTA/66.7% glycerol in a final volume of 20 ml. The DNA was sheared for 30 min at 40,000 rpm in a VirTis homogenizer. A dry ice/isopropanol bath was used for cooling. After ethanol precipitation of the DNA, polymerase I was used to fill in the single-stranded regions generated by shearing (providing the single-stranded projection had a 5′ end). *Bam* linkers (5) were then ligated onto ends of the shear fragments (75 pmol of linkers in a total volume of 20 μl). After digestion with *Bam*, the fragments (now bearing *Bam* sticky ends) were cloned into the *Bam* site of pBR322. Transformed cells were selected for ampicillin resistance and λ immunity (1). DNA was purified (12) from a small number of candidates, and the sequence of the inserted *Bam* piece from the plasmid with the smallest insert (pTR116) was determined (13). The insert extended from the *Hin* II site at the left end of the original restriction fragment to a point five bases past the 3′ end of the *cro* gene (data not shown). The shearing procedure thus neatly separated the *cro* gene from certain λ control elements near the carboxy terminus of the gene that we wished to exclude from our future constructions.

pTR151. pTR116 DNA (5 μg) was partially digested with *Bam*, and those DNA molecules receiving only one *Bam* cut were separated from the other species present by agarose gel electrophoresis. After extraction from the gel (14), this DNA was treated with DNA polymerase I and the four deoxynucleotide triphosphates to render flush the *Bam* sticky ends (1). The resulting molecules were circularized with T4 ligase and used to transform *E. coli*. DNA was isolated from several ampicillin-resistant, λ-immune colonies, and restriction analysis was carried out to determine which of the product plasmids had lost the desired *Bam* site (data not shown).

pTR161. pTR151 DNA (5 μg) was opened with *Bam*. DNA polymerase I was used to fill in the resulting sticky ends. The DNA was then cut with *R* I and the *R* I–*Alu* promoter fragment (in 3-fold molar excess) was ligated into place. All plasmids were constructed under conditions conforming to the standards outlined in the National Institutes of Health guidelines.

Radioimmunoassay. *cro* protein was measured in lysates of transformed cells by radioimmunoassay using the procedure of A. Johnson (unpublished results). Radioimmunoassay measurements were quite precise—there was, on the average, less than 5% difference between duplicate measurements on a culture. The amount of *cro* protein was found to be a function of cell growth conditions. However, measurements made on separate occasions on cultures of independent transformants bearing a given plasmid differed by less than 35%, and the ratio of *cro* protein present in strains bearing two different plasmids varied less than 10% from day to day. Gel electrophoresis of total soluble protein from strains bearing plasmid pTR214 gave estimates of *cro* protein as a percent soluble protein in accord with the radioimmunoassay determination (data not shown) —this indicates that the majority of the *cro* protein detected by radioimmunoassay is intact.

DNA Sequence Analysis. DNA from each of the various λ–*lac* fusion plasmids was digested with *Bgl* II, which cleaves at a unique site 67 bases downstream from the amino terminus of the *cro* gene. The resulting linear DNA was 3′-end labeled by using DNA polymerase I and [α-^{32}P] dGTP and [α-^{32}P] dATP (15). Subsequent *R* I digestion released a small DNA fragment, 162–220 base pairs long, bearing the region of fusion of *lac* and λ sequences. This fragment was isolated by polyacrylamide gel electrophoresis, and the sequence through the fusion region was determined by the method of Maxam and Gilbert (13).

RESULTS

pTR151 DNA was opened with *Bam*, resected with exonuclease III for times ranging from 30 sec to five min, and then treated with S1 nuclease (16). An *R* I–*Alu* fragment bearing the *lac* promoter was ligated into place. After transformation, cell cultures were plated on ampicillin selective plates containing the indicator 5-chloro-4-bromo-3-indolyl-β-D-galactoside. Blue colonies, arising from cells that bear the *lac* promoter on the transforming plasmid and are thus *lac* constitutive, were then checked for *cro* protein production by cross-streaking against phage λ. A typical experiment yielded 400 colonies per μg of pTR151 DNA, 70% of which are blue. Of these blue colonies, 10–90% were immune. Our assumption, later confirmed by radioimmunoassay, was that increasingly high levels of *cro* protein production would confer resistance to ever more virulent derivatives of λ. Clones were tested for immunity to phages bearing no operator mutations (λKH54) or four or more operator mutations (λ4*V'S*, λc411). In this way, the transformants were divided into categories by level of immunity. DNA was isolated from some 40 transformants, representative of the various classes, and characterized by digestion with *Hae* III (see Fig. 2). Because the entire sequence of pBR322 is known (17) as well as the sequences of the *lac* promoter fragment (18) and the *cro* gene itself (19), the *Hae* digests were readily interpreted. The size of the deletion made by exonuclease III and S1 nuclease treatment was estimated from the size of the fragment containing the *lac–cro* fusion.

Finally, nine representative transformants were chosen for more detailed analysis. The level of *cro* protein produced by each of these clones was measured by radioimmunoassay, and the DNA sequence across the *lac–cro* fusion was determined. Fig. 3 shows the results of this analysis.

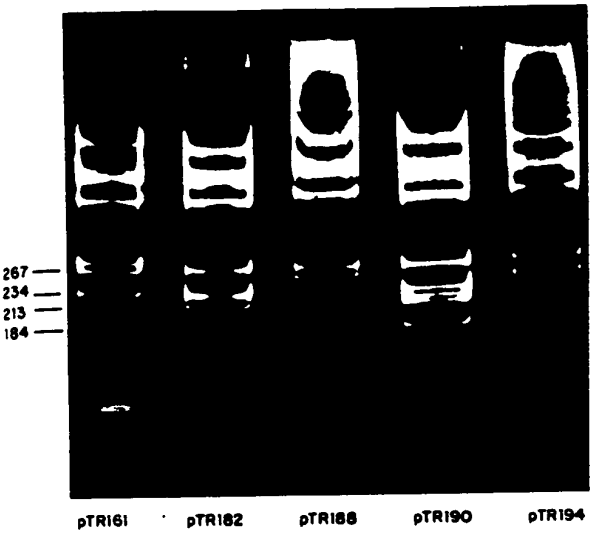

FIG. 2. Analysis of *Hae* III digests of plasmid DNA by gel electrophoresis. Plasmid DNAs from the various *cro* protein-producing strains were partially purified and digested with *Hae* III. Digests were electrophoretically separated on 8% polyacrylamide gels. Of the *Hae* III fragments produced from any plasmid, all but one fragment are common to all plasmids. The one varying fragment contains the region of fusion between *lac* and λ sequences. It can be sized by comparison to the other fragments, the whole sequences of which are all known. The lengths in base pairs of four of these pBR322 fragments are indicated.

DISCUSSION

Strains bearing two of our fusions, pTR213 and pTR214, are rather spectacular overproducers of *cro* protein. The levels of *cro* protein as a percent of total soluble protein shown in Fig. 3 of 1.6% and 1.0% correspond to roughly 190,000 and 120,000 monomers of *cro* per cell. We know of no other *E. coli* strains which produce any protein to a significantly greater extent on a molar basis. If there are 50 copies of pTR213 per cell [the copy number of pBR322 (6)], each *lac* promoter must be directing the production of 4000 monomers of *cro*. This is roughly the fully induced level of expression of β-galactosidase from the same promoter when it directs transcription of the *lac* operon (2). We have not excluded the possibility that these fusions have acquired additional mutations that change the number of copies of plasmid maintained in the cell. The high frequency with which fusions are produced that direct the synthesis of large amounts of protein argues strongly against this possibility, however.

The *lac–cro* fusions presented here represent the beginning of a systematic examination of the effect of gene–promoter separation on protein production. The nine plasmids described in Fig. 3 all carry a complete *cro* gene and a complete *lac* promoter, separated by varying distances. However, the variation in the amount of *cro* protein produced by strains bearing the different fusion plasmids is enormous. For example, strains carrying pTR213 produce over 2000-fold more *cro* protein than strains carrying pTR190. Current theory offers plausible explanations for some of the observed differences, but fails to explain all of them. The same promoter is being used in each plasmid, and so we assume that transcription across the *cro* gene is uniform in each case, and therefore the differences in protein productions are due to differences in some post-transcriptional process. The *cro* mRNAs transcribed from the various plasmids differ in their leader regions, and these differences might affect mRNA stability or processing or ribosome binding efficiency. We do not know how the presence of different leaders might influence the stability or processing of a message. However, current theory holds that a ribosome binding site on a prokaryotic mRNA is composed of two parts: the AUG or GUG signaling the start-point of translation and the so-called Shine and Dalgarno sequence—3–9 bases in the leader portion of the message that are complementary to bases at the 3′ end of the 16S rRNA (21, 22). In six of the nine plasmids, the bases coding for the *cro* Shine–Dalgarno sequence are intact (pTR161, 213, 199, 214, 188, and 194), and it seems reasonable to assume that this sequence is functioning in ribosome binding. Although relatively high levels of *cro* protein are produced in cells bearing all of these plasmids, there are striking differences among them. For example, the deletion in pTR199 is only three bases longer than that in pTR213 and five bases shorter than that in pTR214; yet, strains transformed with pTR199 direct synthesis of 1/10th of the protein of either of the others. In pTR210 one of the bases of the *cro* Shine–Dalgarno sequence has been removed, and yet strains carrying this plasmid produce 10-fold higher levels of *cro* protein than strains carrying pTR199. We have no explanation for these differences. In the two remaining plasmids, the bases coding for the *cro* Shine–Dalgarno sequence are either

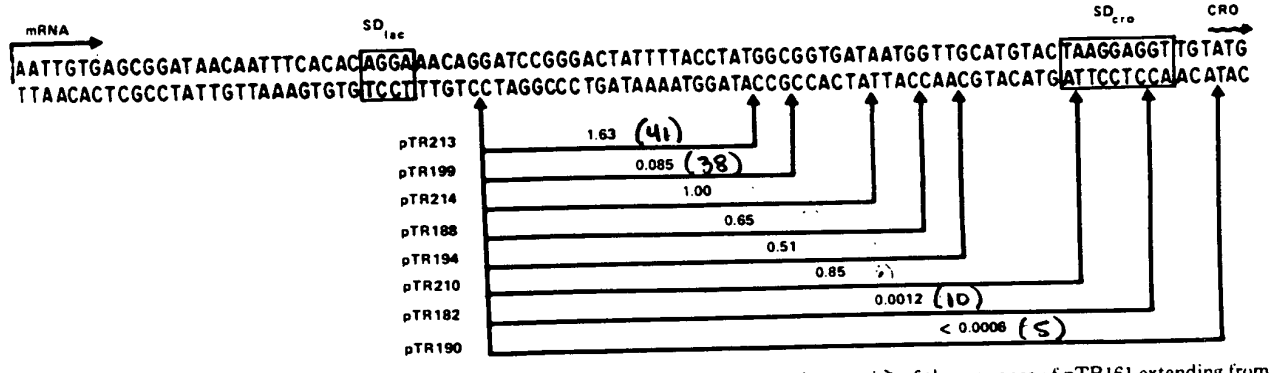

FIG. 3. A summary of the deletion size and *cro* production of selected plasmids. Shown is a portion of the sequence of pTR161 extending from the start-point of transcription of the *lac* promoter on the left (straight arrow) to the start-point of translation of the *cro* gene on the right (wavy arrow). All plasmids produced by the *Exo* III–S1 method described in Fig. 1 may be considered deletions of pTR161. The extent of these deletions is indicated by brackets. The numbers on the brackets are the level of *cro* protein as a percentage of total soluble protein in cells transformed with each of the deletion plasmids. For comparison, *cro* protein represents 0.5% of the total soluble protein in cells transformed with pTR161, and a previously reported plasmid (20) in which *cro* transcription is initiated at its own promoter directs synthesis of 0.05% *cro*. By cross-streaking, strains bearing pTR182 and pTR190 are immune to λKH54; and strains bearing pTR213, 214, 188, 194, and 210 are immune to λKH54, λ4*V*′S, and to λc411, a λ strain selected from λ4*V*′S for growth on *E. coli* strains producing large amounts of *cro*.

largely (pTR182) or totally (pTR190) absent. Cells transformed with these plasmids produce extremely small amounts of *cro* protein. This is perhaps understandable in light of the absence of the *cro* Shine–Dalgarno sequences; however, it must be remembered that presumptive ribosome binding sites in pTR182 and pTR190 are actually coded for by a fusion of *lac* and λ sequences. The *lac* promotor fragment used in the construction of all the plasmids described here contains, in addition to the binding site for RNA polymerase and the start-point of transcription, the coding region for most of the leader of the *lacZ* message including its Shine–Dalgarno sequence. Thus, in pTR182 and pTR190, the *lac* Shine–Dalgarno sequence is brought into close proximity with the ATG of *cro*. We know that such hybrid ribosome binding sites can be extremely effective from the results with pKB280 (2). It is possible that the separation of this sequence and the ATG in pTR190 and pTR182 is not optimum—the *lac* sequence is separated from the ATG of *cro* by 10 bases in pTR182 and by 5 bases in pTR190, whereas the same sequence is separated from the ATG of the *cI* gene by 8 bases in pKB280 and is separated from the ATG of the *lacZ* gene by 7 bases in the *E. coli* chromosome. Work is in progress to produce a *lac–cro* fusion in which the *lac* Shine–Dalgarno sequence is seven bases from the ATG of *cro*.

The method of exonuclease III and S1 nuclease digestion used here should allow the placement of the promoter-containing fragment at virtually any distance upstream from most other genes. As we mentioned earlier, the requirements for restriction sites in and around other genes of interest are extremely flexible. Perhaps the only limitation in promoter placement suggested by the sequence data in Fig. 3 is a tendency of the deletion to stop at As or Gs in preference to Ts in the 5′ (top) strand (there are not enough Cs available to make a judgment). In particular, the technique should be useful in positioning the *lac* promoter fragment adjacent to a eukaryotic gene with the hope of forming a hybrid ribosome binding site from the *lac* Shine–Dalgarno sequence and the ATG of the eukaryotic gene. Expression of the eukaryotic gene could be monitored by radioimmune techniques (23, 24). It remains to be seen whether such fusions will produce eukaryotic proteins in *E. coli*.

We thank A. Johnson for purified *cro* protein and anti-*cro* serum, J. Eliason for phage stocks, A. Jeffrey for restriction enzymes *Hae* III and *R* I, W. McClure for *E. coli* exonuclease III and polymerase I, and R. Scheller for *Bam* linkers.

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occurring during initiation: Since both S1 and properly functioning initiation factors are stringently required for *E. coli* ribosomes to recognize those R17 initiator regions that have weak complementarity to 16 S rRNA (Steitz *et al.*, 1977), it is not surprising that *B. stearothermophilus* ribosomes encounter difficulty interacting with sites other than the highly complementary R17 A protein initiator region. Support for this interpretation comes from the work of Isono and Isono (1975), who were able to achieve novel translation of the f2 coat and replicase cistrons simply by adding *E. coli* S1 to *B. stearothermophilus* ribosomes! We conclude that both proteins and rRNA must be regarded as potential contributors to initiation specificity in prokaryotic systems.

2.6 *mRNA Structure and Initiation*

So far in our discussion of the nature and functioning of initiation signals, we have focused only on limited regions of the mRNA adjacent to authentic initiator codons. However, recognition by ribosomes is also likely to be influenced by intramolecular interactions within an mRNA molecule. For instance, if either the initiator triplet, the polypurine tract, or both of these elements were to be made unavailable for ribosome attachment (for example, by the assumption of a stable RNA secondary or tertiary structure), a potential initiation signal could be rendered inactive. On the other hand, the possibility that RNA conformation might contribute in a positive way to ribosome recognition of initiation sites should not be ignored.

2.6.1 *Evidence for mRNA Secondary and Tertiary Structure*

Certainly the best characterized mRNAs from a structural standpoint are the RNA bacteriophage genomes. Here not only are extensive nucleotide sequences known, but also, because of the availability of large amounts of material, physical–chemical studies of these polycistronic mRNA molecules have been conducted.

The complete nucleotide sequence of the MS2 phage genome (Min Jou *et al.*, 1972; Fiers *et al.*, 1975, 1976) suggests that this mRNA folds into a series of well-defined hairpin loops involving at least 65% of its 3569 residues in Watson–Crick base pairs (see Fig. 2). The existence of the proposed RNA secondary structures is supported both by the results of partial ribonuclease digestion experiments, which yield a characteristic and highly reproducible pattern of oligonucleotide products, and by the observed specificity of chemical mutagenesis performed on whole phage particles (Min Jou *et al.*, 1972). According to the theoretical calculations of Gralla and Crothers (1973), the maintenance of most of these loop structures is also favored in solution under physiological conditions—that is, inside a phage-infected cell or in an *in vitro* protein-synthesizing system. In fact, direct measurements using temperature-jump relaxation (Gralla *et al.*, 1974) and nuclear magnetic resonance methods (Hilbers *et al.*, 1974) of the thermal melting behavior of one specific phage RNA fragment (a 59-nucleotide segment of the R17 genome) yielded T_m values within 5°C of predicted values (see Fig. 3 and Section 2.7.1a). A possible danger in extrapolating from detailed knowledge of the secondary structure of a particular isolated region is that its environment in the intact phage

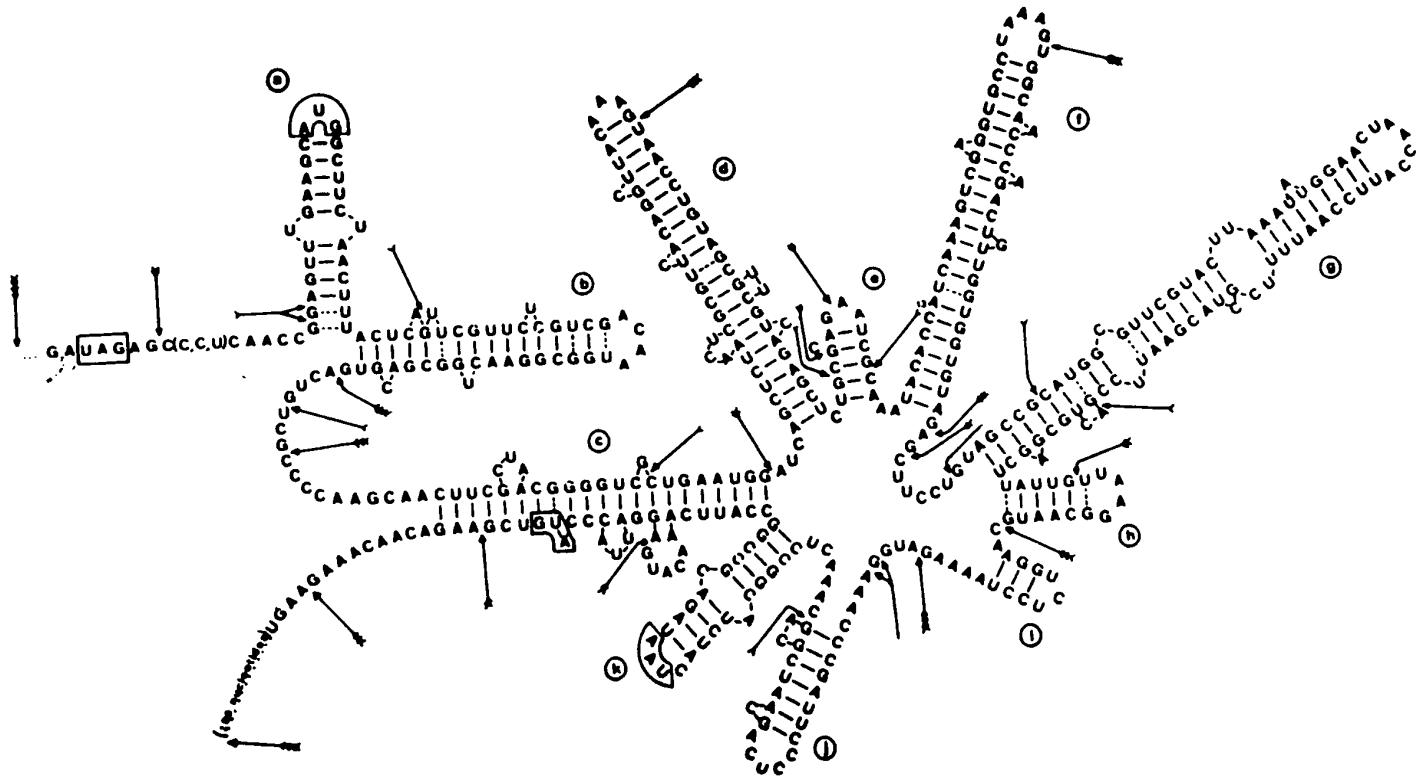

Figure 2. Nucleotide sequence and predicted secondary structure ("flower model") for the coat protein cistron and adjacent regions of bacteriophage MS2 RNA. Terminator triplets (at the ends of the A and coat cistrons) and initiator codons (for the coat and replicase genes) are blocked in. Arrows indicate sites of cleavage of the MS2 RNA molecule by T_1 ribonuclease under conditions of partial digestion; the number of feathers provides a measure of the relative susceptibility of each site, where four feathers indicate bonds always split. (From Min Jou *et al.*, 1972, with permission of the authors and publishers.)

mRNA may provide an opportunity for the formation of alternative conformations that are even more stable. However, since the likelihood of interaction with an adjacent sequence is always higher than with a more distant site, in most cases the MS2 RNA secondary structures that have been proposed are probably correct (Min Jou *et al.*, 1972; Fiers *et al.*, 1975, 1976).

Assuming that the phage RNA does undergo extensive local helix formation, we can further ask whether such structures associate in any preferred way to yield a defined tertiary conformation. Evidence here is much less direct, but nonetheless persuasive. Even the earliest physical–chemical measurements on phage RNA molecules indicated not only a high degree of helicity (60–80%) but an unusually compact overall structure (for a review see Boedtker and Gesteland, 1975). The observation that various ribonucleases first cleave the RNA phage genome within a limited region located about 40% from the 5′ end (again see Boedtker and Gesteland, 1975) is most reasonably explained by a folding pattern which renders this site particularly accessible. Moreover, even at very low salt concentrations, MS2 RNA molecules spread on an electron microscope grid reproducibly display interactions involving distant portions of the genome (Jacobson, 1976). Whether or not these structural features are a consequence of the requirement for the phage RNA to fold into a minimum-sized sphere capable of being encapsulated by a protein coat, the concept of higher order organization in these RNAs is most helpful in explaining many of the translational phenomena discussed later.

In the case of messenger RNAs other than the RNA phage genomes, direct evidence for the formation of preferred three-dimensional structures is sparse. The best data are from experiments with mRNAs isolated from phage T4-infected cells (Ricard and Salser, 1974, 1975); melting studies and partial nuclease digestion experiments suggest a less compact tertiary folding than in R17 RNA. Nonetheless, the extent of T4 mRNA secondary structure appears comparable to

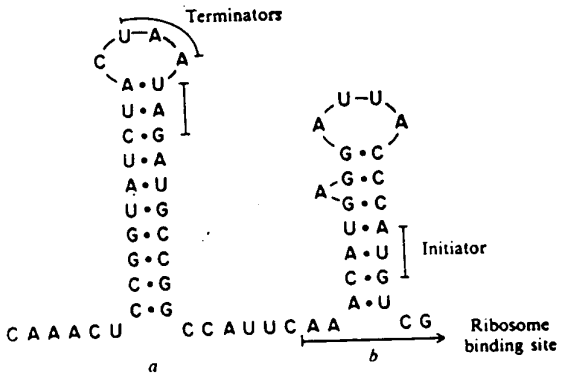

Figure 3. The segment of bacteriophage R17 RNA which is protected from RNase digestion by coat protein (Bernardi and Spahr, 1972; Gralla *et al.*, 1974). It includes an entire intercistronic region and portions of the coat and replicase genes, as indicated. Temperature-jump relaxation studies (Gralla *et al.*, 1974) yielded T_m values of 83 and 61°C for hairpin loops *a* and *b*, respectively. Binding of coat protein to the fragment alters the melting behavior of helix *b* only. (Reproduced from Gralla *et al.*, 1974, by permission of the authors and publisher.)

rRNA, from which reproducible oligonucleotide fragments indicative of the existence of many defined hairpin loops have been obtained during sequence analysis (Ehresmann *et al.*, 1975). The limited work to date on bacterial mRNAs suggests that they may undergo significantly less internal base pairing, which could be related to their short half-lives *in vivo*. However, since even totally random RNA sequences are predicted to form secondary structures involving about 50% of their residues in base pairs (Fresco *et al.*, 1960; Gralla and Delisi, 1974), it seems unlikely that bacterial mRNAs have evolved to resist any loop formation under all physiological conditions. Again, perhaps the best argument for the existence of secondary structures in non-RNA phage messengers is that mRNA folding can provide plausible molecular explanations for otherwise puzzling behavior; several examples are discussed in the sections on translational control and reinitiation.

2.6.2 *Secondary Structure Prevents False Starts in RNA Phage Messengers*

Since the length of the region complementary to 16 S rRNA in authentic messenger initiation sites can be as short as three nucleotides (Table I), it is obvious that the potential for base pairing plus the presence of an initiator triplet is not sufficient to describe a true mRNA initiator region. Indeed, a quick scan of nucleotide sequences in the MS2 RNA phage genome (Min Jou *et al.*, 1972; Fiers *et al.*, 1975, 1976) reveals a number of internal and out-of-phase AUG and GUG triplets that are preceded by appropriately situated polypurine tracts. Yet, only the three regions listed in Table I are recognized by *E. coli* ribosomes as functional start signals in the intact RNA molecule. If secondary structure does play an active negative role in limiting initiation to the proper regions, we would expect that sequences surrounding each of the additional potential initiators should be at least partially sequestered by RNA secondary structure. In fact, examination of Fig. 2 and the MS2 A (Fiers *et al.*, 1975) and replicase (Fiers *et al.*, 1976) cistrons shows this to be largely true.

Substantial experimental evidence can also be marshalled to support the notion that the RNA phages utilize secondary structure to restrict initiation. Most compelling is the finding that partial unfolding of the MS2 or f2 RNA molecule with formaldehyde (which has been shown to disrupt base pairing) (Feldman, 1973) results in the synthesis of a discrete set of novel fMet-dipeptides (Lodish, 1970*b*) that can be elongated to produce a heterogeneous population of polypeptide products. Although no sequence analysis has yet been undertaken on these "counterfeit" initiator regions, translation presumably originates at internal and out-of-phase initiation triplets that are normally blocked. Interestingly, utilization of these sites is independent both of the presence of S1 protein on the ribosome (van Dieijen *et al.*, 1976) and of initiation factor IF-3 (Berissi *et al.*, 1971), suggesting that their complementarity to 16 S rRNA may be rather strong. Evidence that RNA secondary, rather than tertiary, structure is responsible for inactivating these internal initiators comes from the observation that fragmentation of the phage RNA does not yield new fMet-dipeptides until the fragments approach 4S in size (less than 100 nucleotides) (Staples and Hindley, 1971; Steitz, 1973*b*; Voorma *et al.*, 1971). Furthermore, statistical analysis of the differences in nucleotide sequence among the group I RNA phages (MS2, f2, R17) suggests that there has been selective pressure to preserve the RNA secondary structures (Min

Jou and Fiers, 1976). Whether messenger RNAs other than the RNA phage genomes have evolved a similar mechanism for limiting translational initiation is not yet known.

2.6.3 *Structural Effects at Authentic RNA Phage Initiation Sites*

Extensive *in vitro* manipulation of the RNA phage genome has also provided excellent evidence that three-dimensional RNA structure effects a significant decrease in the rate at which *E. coli* ribosomes attach to two of the three true phage initiator regions (for reviews see Lodish and Robertson, 1969; Steitz, 1975; Lodish, 1975). With intact RNA from either the group I phages or $Q\beta$, protein synthesis is initiated efficiently at only one of the three ribosome-binding sites, that of the coat cistron (for a review see Kozak and Nathans, 1972). Replicase initiations occur at about one-third this rate and are dependent on prior translation of a portion of the coat protein cistron (the so-called polarity effect; see later). A protein is synthesized at only about one-twentieth the rate of coat protein. At least three treatments, all of which are predicted to disrupt the native secondary/tertiary conformation of the RNA molecule, stimulate both replicase and A protein synthesis dramatically. These are mild fragmentation (for references see Steitz, 1973*b*), heat treatments under various ionic conditions (Fukami and Imahori, 1971), and formaldehyde unfolding (Lodish, 1970*b*, 1971). Since the newly exposed authentic initiator regions are bound by either *E. coli* or *B. stearothermophilus* ribosomes in accord with the documented species-specificity pattern (see Section 2.5), apparently only the availability of the sites for ribosome attachment is being altered. Accordingly, it has also been observed that nascent RNA molecules are significantly better messengers for both f2 (Robertson and Lodish, 1970) and $Q\beta$ (Staples *et al.*, 1971) A protein synthesis (the A cistron is the first gene) than are mature RNA molecules. This finding is consistent with the idea that A protein initiations are severely limited in the intact phage mRNA. In fact, Fiers *et al.* (1975) have recently noted that a nucleotide sequence appearing two-thirds of the way along the A cistron in MS2 RNA can base-pair with approximately 15 residues just preceding the A protein initiator triplet; an interaction between these two regions could be that which restricts initiation.

Experiments which examined the ability of isolated RNA phage initiator regions to rebind to ribosomes further suggest a positive contribution of RNA structure to initiation at some sites. Whereas the R17 A protein initiator region becomes an exceedingly efficient initiation site when it is released from the remainder of the molecule (Steitz, 1973*b*), surprisingly the region at the beginning of the R17 coat protein cistron decreases in initiation potential upon release (Adams *et al.*, 1972; Steitz, 1973*b*). Two molecular explanations are possible: Either the coat protein site folds into a stable secondary structure that precludes ribosome binding when it is an isolated fragment but not when it is in the intact molecule, or the overall tertiary structure of the phage RNA may be designed such that this region is normally so exposed that it cannot be ignored by a passing ribosome. Long-range positive effects of RNA structure also seem to operate at the $Q\beta$ coat protein initiator; here it has been found that fragments containing this region do not rebind detectably to ribosomes unless they are at least 100 nucleotides long (Porter and Hindley, 1973).

ribosome-binding-site *a* (Table I) and transforms its five-base match with 16 S rRNA (GAGGU) into a less complementary sequence (GAAGU). Concomitantly, a drop of about tenfold in the efficiency of synthesis of the gene *0.3* protein in T7-infected cells is observed. This important observation provides direct evidence that mRNA·rRNA base pairing makes a significant contribution to ribosome recognition of true initiation signals in mRNA *in vivo*.

Finally, if RNA secondary structure is indeed a potent negative regulator of initiation, both "up" and "down" mutations that alter the ability of the mRNA region surrounding an initiator triplet to assume a stable secondary conformation should be discovered. A most intriguing possibility (Singer and Gold, 1976; Belin and Epstein, 1977) is HD263, a recessive temperature-sensitive mutation which affects the synthesis of the bacteriophage T4 rIIB protein. At 25°C, the amount of mutant protein produced is 10 to 20% of the wild type level; at 37°C and above, no detectable synthesis occurs. Reduced transcription, messenger instability, and differences in protein sequence or degradation have been ruled out as possible explanations. Rather, the existence of a "melting curve" for rIIB expression in HD263 suggests that RNA conformation plays a role in translational initiation at this site. Presumably, sequence analysis of the region surrounding the beginning of the rIIB cistron will establish whether this intuition is correct.

2.10 *Perspectives and Problems*

If now asked to summarize how a ribosome recognizes a true initiation signal in messenger RNA, we can come up with the following molecular picture. The mRNA primary structure participates in at least two RNA·RNA interactions during the positioning of the ribosome to form the initiation complex: specific portions of the message base pair with the initiator tRNA on the one hand and with the 3′ end of the 16 S rRNA on the other. Also, during the ribosome-binding process, mRNA secondary structure functions as a controlling element, covering unwanted internal initiators and regulating the availability of authentic initiation sites. However, simple accessibility of an mRNA region including an initiator triplet and a sequence complementary to 16 S rRNA is not sufficient to define an initiator region; we have seen that *B. stearothermophilus* ribosomes fail to recognize several RNA phage sites that are bound by *E. coli* ribosomes under identical conditions. Thus, proteins, several of which can be viewed as acting through the two RNA·RNA interactions, enter the picture as additional contributors to mRNA recognition by ribosomes. Not bad for just the ribosome and the mRNA!

Considering what defines an active initiator region inside a living cell brings up numerous complications. In addition to variables such as the level of transcription and messenger lifetime, there exists the possibility of competition between mRNAs. Under circumstances in which ribosomes are limiting, presumably those initiator regions whose individual interactions sum to give the highest ribosome affinity will win out over weaker sites (for an analytical treatment see Lodish, 1974). Further, we have seen that some initiator regions are designed to be sensitive to the intracellular concentration of a regulatory protein, whose binding effectively eliminates its target site from the competing pool. Certain temporal factors must also be important *in vivo*. For instance, all buried internal codons

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SECONDARY STRUCTURE OF mRNA AND EFFICIENCY OF TRANSLATION INITIATION

(Recombinant DNA; *lac* promoter; λ *cro* protein; *E. coli* plasmid deletions; RNA folding)

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SUMMARY

A series of recombinant plasmids, containing the *cro* gene of phage λ, exhibit strikingly different levels of expression depending apparently only on the nucleotide sequence of the untranslated 5′ mRNA (Roberts et al., 1979). We postulate that initiation of translation involves interaction between an activated 30S ribosomal subunit and the 5′-terminal region of a messenger RNA already folded in a specific secondary structure. The observed variation in *cro* synthesis can then adequately be explained by secondary structure models which were derived for the different mRNAs. To maximize expression, it appears necessary that the initiation codon and, although less important, the ribosome interaction site are accessible.

INTRODUCTION

Initiation of translation on natural mRNAs is one of the most essential processes in the cell but is still not understood in detail. In prokaryotes, gene translation can start either with an AUG or a GUG codon, but this is obviously not a sufficient signal. A purine-rich sequence called the Shine and Dalgarno sequence, for which the prototype is AGGAGGU (Shine and Dalgarno, 1974), preceding the initiation codon usually by 6 to 8 nucleotides (Godson et al., 1978; Steitz, 1979), has been implicated in binding of the 30S ribosomal subunit to the beginning of the gene. But at least one mRNA is known to start directly with an initiating AUG for translation (Walz et al., 1976; Ptashne et al., 1976). Moreover, in the RNA phage genome many regions are present which, if considered in terms of primary nucleotide sequence alone, fulfill all criteria for an optimal ribosome binding

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region yet are not functional as translation starts in vivo. Obviously, additional factors determine the specificity and the efficiency of initiation of translation [for recent reviews see Steitz (1979) and Fiers (1979)].

In order to address this problem Roberts et al. (1979) have isolated a set of nine recombinant plasmids which contain the complete *cro* protein gene of phage λ preceded by the *lac* promoter of *Escherichia coli*. These plasmids differ only in the 5'-terminal leader sequence preceding the *cro* gene. In the prototype plasmid (Fig. 1a), the leader RNA consists of the 36 nucleotides of the *lac* message fused to the 58 nucleotides of the λ message, preceding the *cro* initiating AUG. In the derivatives, deletions have been introduced starting at nucleotide 37 and extending towards the initiating AUG. The enormous differences in the amount of *cro* protein produced by these plasmids does not simply correlate with the length of the deletions. So far no satisfying explanation for this wide variation in expression has been provided. It is unlikely that these differences could be caused by the regulatory effects of the *cro* protein, since the entire o_R3 operator and half of the o_R2 operator are deleted, or by the variable copy number of plasmids per cell (Roberts et al., 1979).

In the present study we propose a series of secondary structure models for the 5'-terminal region of these different *cro* mRNAs. The involvement of the initiating AUG codon and of the ribosome interaction site in the secondary structure can explain adequately nearly all the observed differences in efficiency of translation. (In our terminology "ribosome interaction site" refers to a Shine and Dalgarno sequence on the mRNA which can directly basepair with a segment near the 3' end of 16S ribosomal RNA; "ribosome binding site" is the region on the mRNA which in the initiation complex is protected by the ribosome against nuclease digestion.)

RESULTS AND DISCUSSION

Construction of secondary structure models

A secondary structure model for each of the *cro* mRNAs was constructed based on the DNA sequence data of the 5'-proximal RNA (Roberts et al., 1979) including the *cro* gene (Schwarz et al., 1978). The thermodynamic stability of each structure (summarized in Table I) was estimated following the rules of Tinoco et al. (1973). Stability calculations based on the basepairing and stacking values of Borer et al. (1974) confirmed the deduced stability differences. When two hairpins occurred next to each other in a flower-like structure so that we had to choose between considering this part of the model as an interior loop or as a bulge (e.g., Fig. 1a, helices b and c), we always used the former structure for estimating the thermodynamic stability. The destabilization of the base of a helix occurring in an interior loop (e.g., Fig. 1a, helix a) was considered as equal to the destabilization of four nucleotides. Except for the *cro* mRNA of plasmid pTR190, which has the largest deletion (extending to just before the initiating AUG codon), all the

TABLE I

EXPRESSION OF THE *cro* PROTEIN GENE CONTAINED IN A SERIES OF NINE RECOMBINANT PLASMIDS AND THERMODYNAMIC PARAMETERS OF THE SECONDARY STRUCTURE OF THE 5'-PROXIMAL REGION OF THE *cro* mRNA

The plasmids differ only in the distance between the phage λ *cro* gene and the *E. coli lac* promoter inserted in front of the *cro* gene. Production of *cro* protein is given in the second column as a percentage of the total soluble protein content of cells transformed by the respective plasmids (data from Roberts et al., 1979). The next two columns give the stability of two alternative secondary structure models that can be deduced for the 5'-proximal region of the different plasmid *cro* mRNAs with the initiating AUG codon located either in a single-stranded region (third column) or in a double-stranded region (fourth column); the last column gives the energy needed to release the ribosome interaction site (i.e., the Shine and Dalgarno (1974) sequence). Estimates for the thermodynamic stability of stabilizing features (hairpins) and destabilizing features (bulges, loops) of the secondary structures are as reported by Tinoco et al. (1973); as an example, the stability calculation of the structure shown in Fig. 1a is based on the following values. Hairpin a: stabilizing interactions, −32 kcal; destabilizing features, +21 kcal; net −11 kcal. Hairpin b: stabilizing interactions, −39.6 kcal; destabilizing features, +18 kcal; net −21.6 kcal. Hairpin c: stabilizing interactions, −47 kcal; destabilizing features, +22 kcal; net −25 kcal. Stem interaction: stabilizing interactions, −8.2 kcal; interior loop, +3 kcal; net −5.2 kcal. Hence, the overall stability estimate amounts to −62.8 kcal/mol.

Plasmid	*cro* protein production	ΔG of *cro* mRNA structure model with		ΔG for release of the ribosome interaction site[a]
		AUG in a single-stranded region	AUG in a double-stranded region	
			(kcal)	
pTR161	0.5	*−62.8*	−61.8	+14.8
pTR213	1.63	*−56.8*	−56.3	+14.8
pTR199	0.085	−56.6	*−59.5*	+ 3.2
pTR214	1.00	*−52.8*[b]	−52.6	+ 7.4
pTR188	0.65	*−52.8*	−51.8	+14.8
pTR194	0.51	*−49.4*	−48.4	+14.8
pTR210	0.85	*−43.0*	−42.4	+12.4[c]
pTR182	0.0012	−42.0	*−43.2*	—[d]
pTR190	0.0006	—[e]	*−39.4*	—[d]

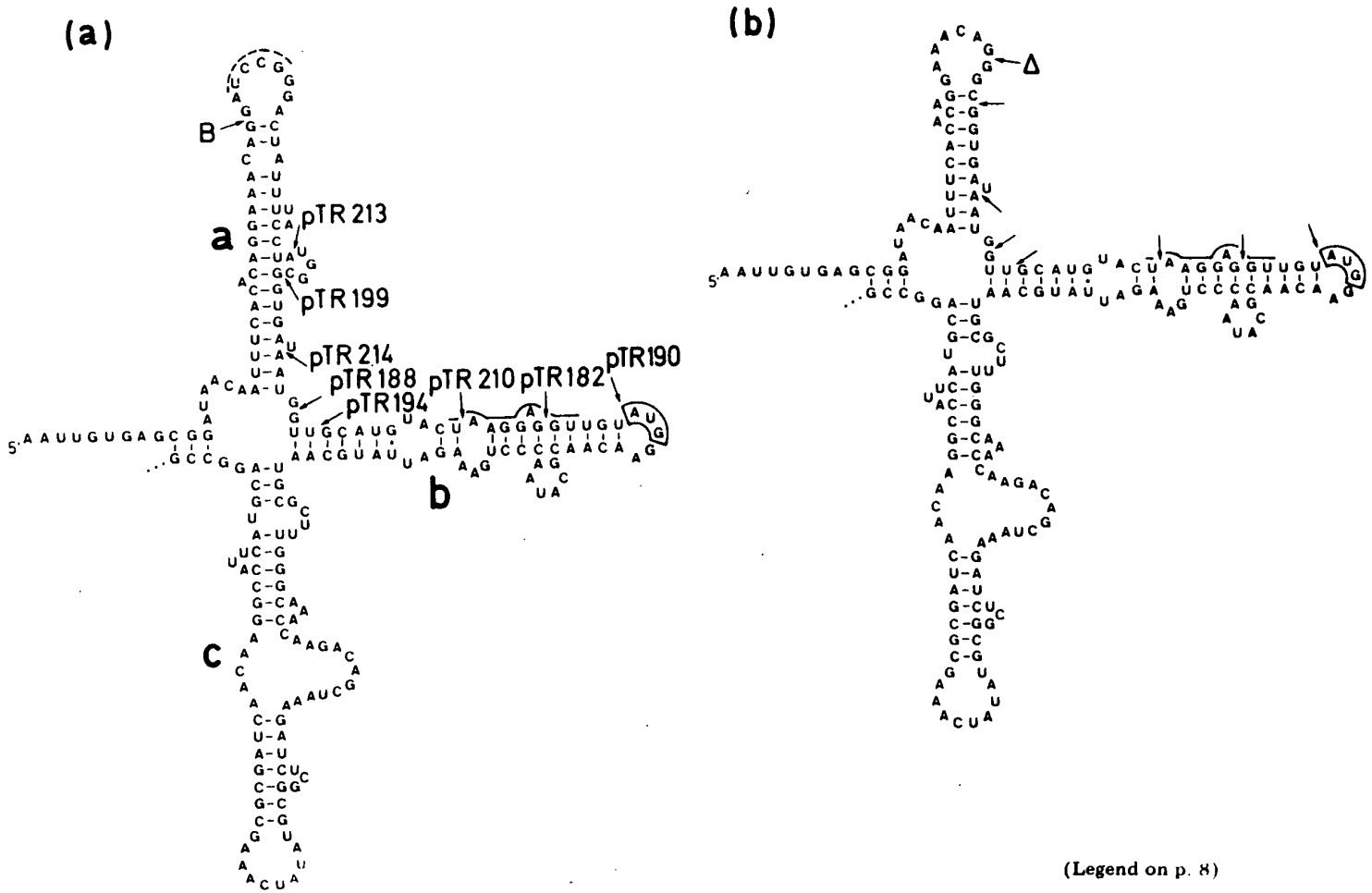
[a] Loss of free energy as a result of the release of the ribosome interaction site (UAAGGAGGU). If the helix becomes unstable by this operation, further interactions are opened up to the most stable conformation that can be achieved without rearranging the overall structure.

[b] An alternative model can be drawn that is as stable as the proposed structure and which is similar to that of pTR188 *cro* mRNA. We preferred the model shown in Fig. 1d because the alternative model has a very large (20 nucleotides) and asymmetric loop which probably is more destabilizing than that predicted by the rules of Tinoco et al. (1973).

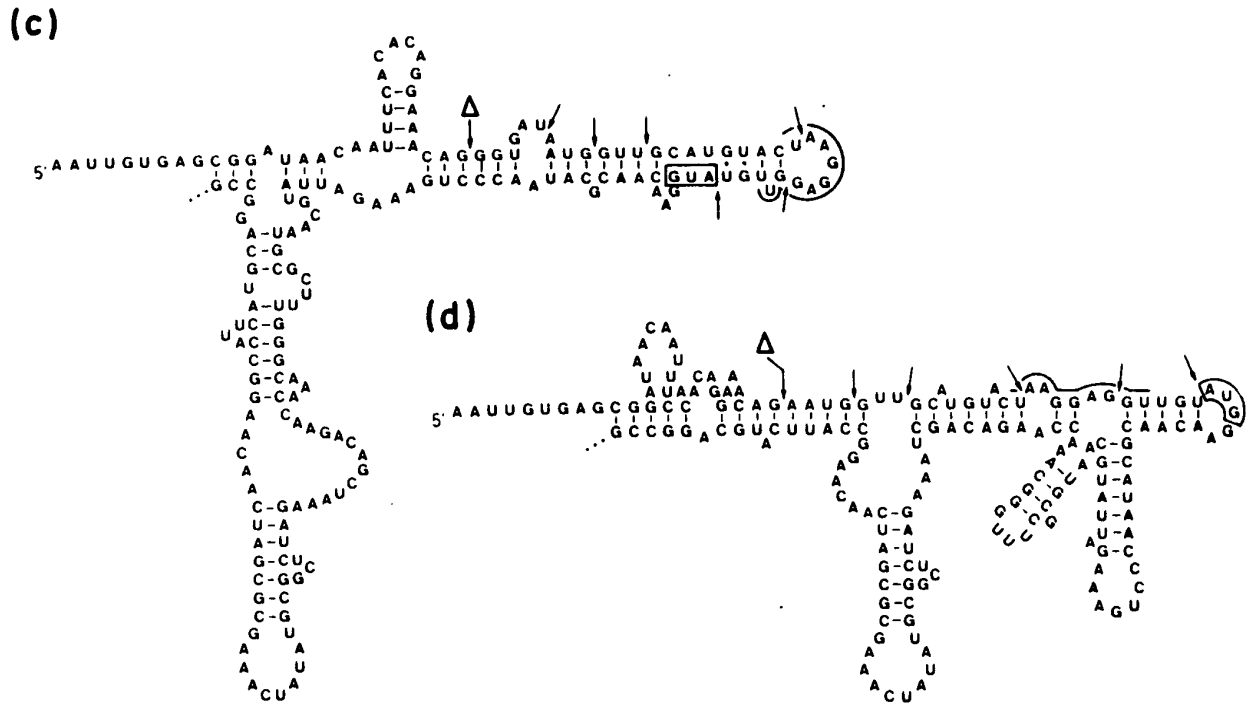
[c] Energy required to release the ribosome interaction site (AAGGAGGU). Note that this sequence is one nucleotide shorter than the ribosome interaction sequences of the preceding plasmid mRNAs.

[d] The DNA coding for the original ribosome interaction site is deleted from these plasmids.

[e] No equivalent model with an accessible AUG can be drawn.


(a)
(b)
B
a
b
c
pTR 213
pTR 199
pTR 214
pTR 188
pTR 194
pTR 210
pTR 182
pTR190
Δ

(Legend on p. 8)



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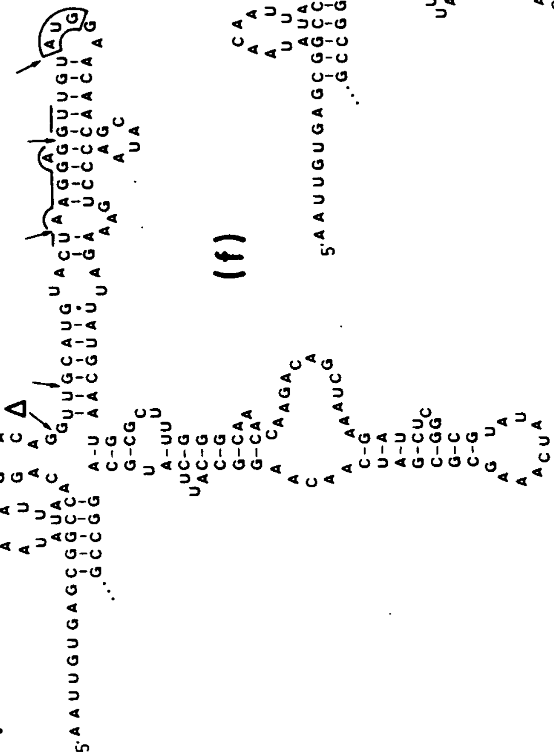
(e)



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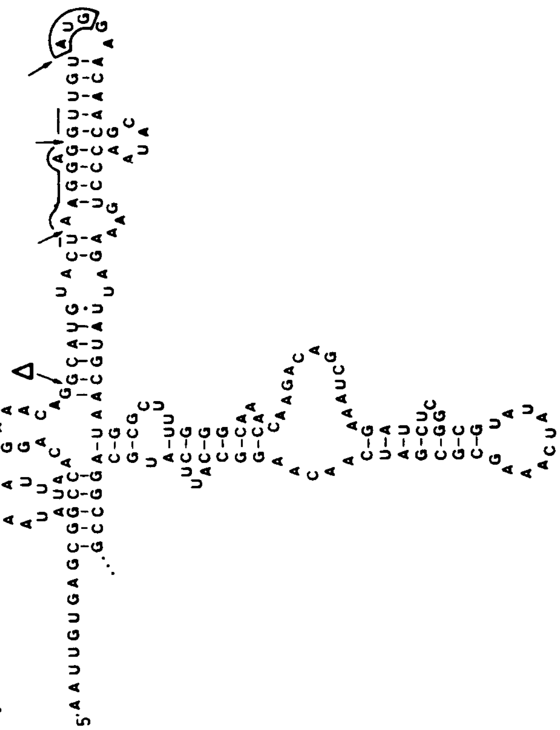

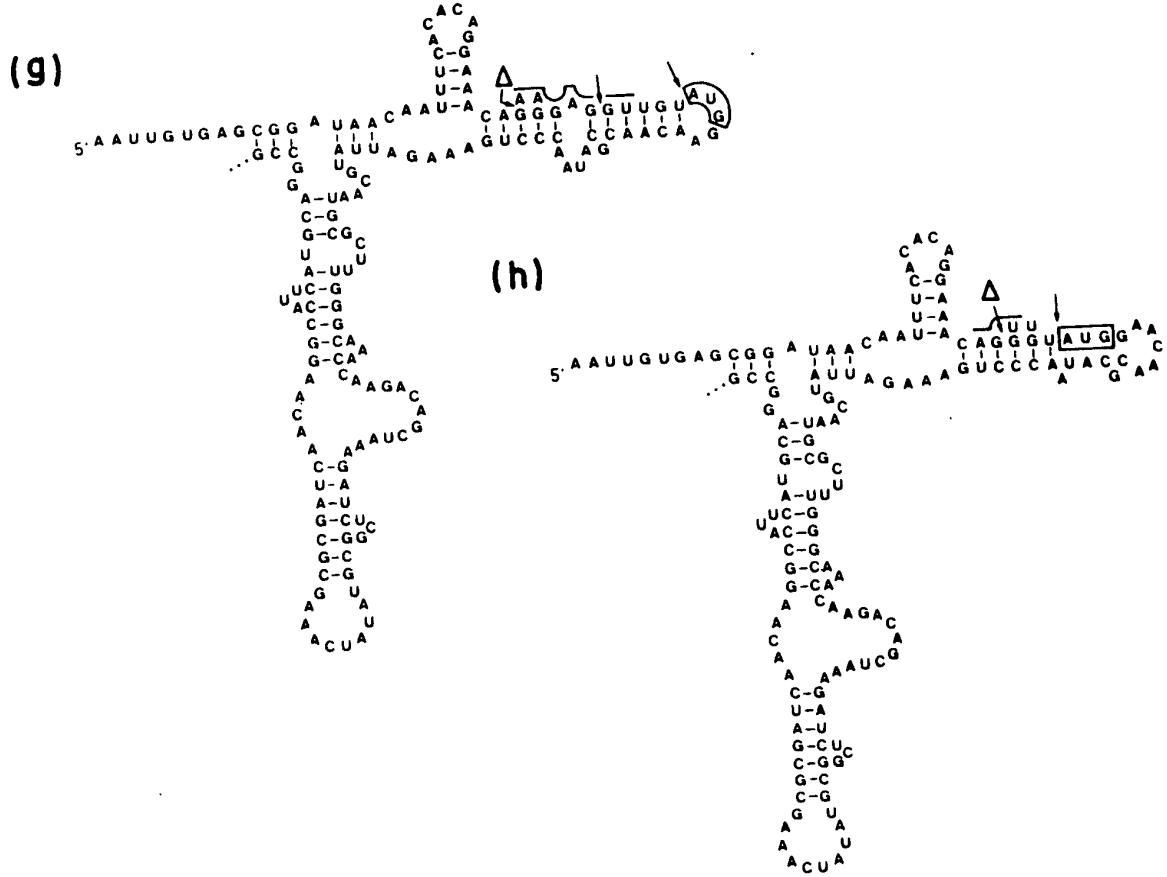

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(i)

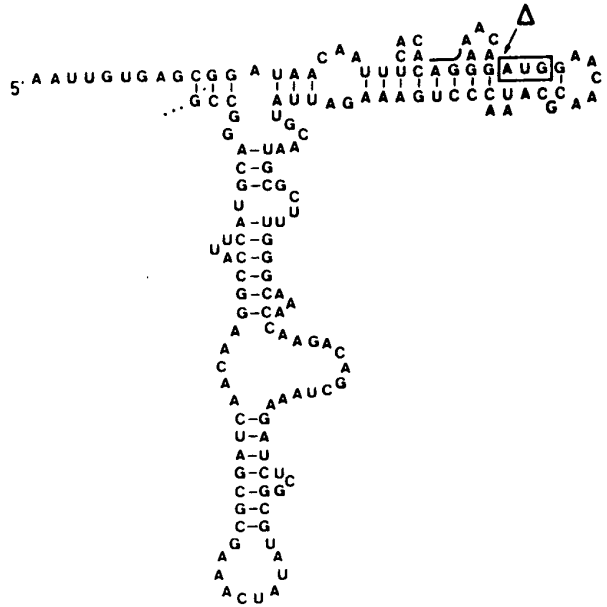

Fig. 1. The most stable secondary structure models of the 5′-terminal segment (including the untranslated 5′-terminal RNA and up to 110 nucleotides beyond the AUG initiation codon) of the *cro* mRNAs corresponding to the recombinant plasmids described by Roberts et al. (1979). (a) plasmid pTR161, (b) pTR213, (c) pTR199, (d) pTR214, (e) pTR188, (f) pTR194, (g) pTR210, (h) pTR182, (i) pTR190. All sequences start off with 36 nucleotides of *lac* mRNA. The B in structure 1a indicates the start point of all the deletions and the arrows indicate the ends of all the pTR deletions; the dashed line indicates the sequence that can partially mask the initiation codon by a tertiary interaction between the loops of helices a and b. In the following secondary structure models the position of the deletion is indicated by a delta symbol. In all the structure models the ribosome interaction sequence is indicated by a solid line.

remaining mRNAs showed two nearly equally stable models, one with the initiating AUG in a hairpin loop and the other with the AUG involved in secondary structure (see Table I for energy values). The most stable secondary structure of each *cro* mRNA is shown in Fig. 1a to i.

Location of the initiation codon in the secondary structure

Table I summarizes the level of *cro* protein production for each plasmid as a percentage of total soluble protein in cells transformed by the plasmid (data taken from Roberts et al., 1979). In the third and fourth column the expected thermodynamic stabilities of the two alternative structures are indicated. On this basis it can be deduced whether the AUG is likely to be located in a hairpin loop or be involved in a base-paired region. Cells transformed by plasmids pTR199, pTR182 or pTR190, where the thermodynamically most stable *cro* mRNA model shows the initiation codon sequestered in secondary structure (Fig. 1c, h and i, respectively), all produce extremely small amounts of *cro* protein, in contrast to the rather high yield in cells transformed by one of the remaining plasmids,where the AUG is freely accessible in a hairpin loop.

Correlation between the minor differences in expression and the secondary structure

Cells transformed by plasmid pTR213 produce about three times more *cro* protein than cells transformed by pTR161, although the *cro* mRNA structure models are similar (Fig. 1b and a, respectively). However, the pTR161 model shows a possible interaction between 5′ . . .U-C-C-G. . . 3′ of loop a and 5′ . . .U-G-G-A. . . 3′ of loop b. If this tertiary structure interaction would indeed occur, it would result in a partial protection of the AUG initiation codon. This interaction is no longer possible in pTR213 *cro* mRNA, where the AUG remains completely accessible.

The AUG in *cro* mRNA of pTR214, pTR188, pTR194 and pTR210 is also situated in a hairpin loop; however, the expression value of pTR214 is nearly twice as high as that of pTR194, whereas pTR188 and pTR210 have intermediate values. For this series, it is of interest to consider the energy needed to release the Shine and Dalgarno type ribosome interaction site from the secondary structure (Table I, column 5). The plasmids pTR214, pTR210 and the couple pTR188 and pTR194 show a striking correlation between the increasing energy needed to free the interaction site and the amount of *cro* protein produced. Only the small difference in *cro* production between pTR188 and pTR194 cannot be explained in this way.

The reason for the difference in expression between pTR213 and pTR214 is not so obvious. It is possible that the first helix of pTR213 (Fig. 1b) has some importance, e.g., in stabilizing the messenger or as a structural signal for ribosome recognition. Cannistraro and Kennell (1979) proposed that the secondary structure in the region preceding the ribosome binding site of the β galactosidase RNA could affect the initiation of translation. They sup-

posed, however, a negative influence, the secondary structure at the 5′ end being a hindrance to ribosome loading. But the thermodynamic stability of the hairpin which these authors propose is rather weak and is not preserved in our models. In any event, the function in translation or in mRNA stability of such a helix which precedes the ribosome binding site remains hypothetical at present.

The plasmids pTR199, pTR182 and pTR190, in which the initiating AUG in the mRNA is sequestered in secondary structure, all exhibit low levels of *cro* expression. Nevertheless, the residual level in the last two is more than an order of magnitude lower compared to pTR199. As only the latter has retained the intact ribosome binding region (this refers not only to the Shine and Dalgarno sequence, but also to the following nucleotides) of the original *cro* gene, this in itself could provide the explanation (Roberts et al., 1979). However, it should be noted that the nucleotide sequence preceding the initiating AUG in pTR182, although hybrid (*lac-cro*), still allows stable interaction with the 30S ribosomal subunit and is not unlike some other ribosome binding regions of active genes (Steitz, 1979). A possible explanation for the drop in expression between pTR199 and pTR182, however, lies again in the higher energy needed to release the ribosome interaction sequence of the latter plasmid. In the model proposed for pTR199 (Fig. 1c), the Shine and Dalgarno interaction site is largely single stranded, and only 3.2 kcal are needed to free it completely (Table I). On the other hand, for pTR182 11.1 kcal are required to release the ribosome interaction sequence, which, moreover, extends over only four base pairs. The low level of *cro* expression by pTR190 compared to pTR199 could be due to the same reason and/or the fact that the *cro* sequence preceding the initiating AUG is totally replaced by *lac* message.

Importance of mRNA secondary structure

The striking correlation between the features of the proposed structure models in Fig. 1a to i and the level of *cro* protein production strongly suggests that the secondary structure of the mRNA plays a major role in initiation of protein synthesis. Our observations indicate that the initiation codon cannot be recognized immediately after its transcription, and it seems that at least a part of the secondary structure must form before ribosome binding takes place. However, a full-length mRNA is not required, as most of the two alternative models for each plasmid mRNA (with the AUG free or with the AUG buried) contain identical helices, and our conclusions remain valid even when as little as 48 nucleotides beyond the initiating AUG participate in the specific folding interactions. Only in the case of pTR213 *cro* mRNA is synthesis of up to 110 nucleotides beyond the initiating AUG required (up to the C-C-G sequence; see Fig. 1b) in order for the preferred secondary structure to form. Although loading of nascent mRNA with ribosomes is generally considered to be a fast process, it may nevertheless be kinetically slow relative to the local formation of secondary structure.

The crucial role of secondary structure in the initiation of translation is in complete agreement with the long standing observation that in bacteriophage MS2 RNA many non-functional ribosome-binding-like regions are present (De Wachter et al., 1971; Fiers et al., 1976; Fiers, 1979). If considered only in terms of primary sequence, many of these closely resemble one or another bona fide ribosome binding region of a bacterial or phage gene. Undoubtedly, secondary structure keeps them silent, and partial unfolding, e.g., by formaldehyde treatment, is sufficient to activate a number of these sites (Lodish, 1970). A similar observation was made by Steege (1977) for the lactose repressor messenger of *E. coli*, where some of the possible reinitiation sites seem to be masked by secondary structure.

The major role of a free initiation codon for functional ribosome binding has recently also been demonstrated in the Qβ RNA system (Taniguchi and Weissmann, 1979). In the absence of formylmethionyl-tRNA, ribosomes bind to three different regions, none of which is in close proximity to a protein initiation site. It follows that a Shine and Dalgarno sequence alone is insufficient to direct correct placement of the ribosome. This agrees with our observation that a free initiation codon is a major factor whereas the involvement of the Shine and Dalgarno site in secondary structure apparently causes a far smaller reduction in *cro* protein production.

The secondary structure models which we have derived for the various plasmid mRNAs provide a satisfactory explanation for the observed levels of translation efficiency. Further proof, however, for the underlying hypothesis is needed, and could be provided, e.g., by the examination of the effect of a series of point mutations on translation and on mRNA secondary structure. A prediction of the model is that mutations which affect initiation of translation efficiency are expected to occur not only in the area preceding the initiating AUG, but also in the first part of the coding region of the gene. Of course the secondary structure fulfills many roles, not only optimizing the initiation of translation, but also modulating the functional life-time of the mRNA, masking pseudoinitiator sequences, etc.

Finally, it may be noted that the effects of mRNA secondary structure on translation as proposed in the present paper are of particular relevance for expression of a cloned foreign gene. Not only a proper start of transcription and a ribosome interaction site have to be provided, but furthermore the accessibility of the initiation sequence including the AUG (or GUG) in the secondary structure must be considered, and this may among other factors depend on the nucleotide sequence of the beginning of the coding region.

ACKNOWLEDGEMENTS

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Communicated by H.G. Zachau.

Gene, 9 (1980) 13—25

DNA REARRANGEMENTS IN A HYBRID PLASMID CARRYING THE *red* B *imm* REGION OF COLIPHAGE LAMBDA

(Insertion elements; illegitimate recombination; recombinant DNA; deletions; restriction endonuclease; *E. coli*; thermosensitive repressor; λ immunity)

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SUMMARY

The hybrid plasmid consisting of pSC101 and the *red*B—*N*—*imm* region of phage λ *c*I857 persists in cells grown at 30°C but not in cells grown at 37°C. In the latter case the plasmid was found to undergo several modifications.

Restriction maps of these new plasmids indicate the following modifications: (1) the insertion of an IS*1* element into gene *N* carried by the λ fragment; (2) a mutation in the p_L o_L site of the same fragment, and (3) four large deletions (30 to 50% of the hybrid plasmid) which remove almost the entire λ fragment. For the latter deletions, one endpoint seems to be fixed in the same restriction fragment of pSC101 while the other endpoint assumes four different positions on the λ fragment; this might suggest a site-specific recombination event.

INTRODUCTION

We previously found that cloning of the *Eco*RI fragment f2 of the phage λ*c*I857 (Allet et al., 1973) into the vector pSC101 (Cohen et al., 1973) was possible only when the transformed cells were grown at 30°C (Bernardi and Bernardi, 1976). Since the f2 fragment carries the gene *c*I857 coding for the thermosensitive repressor of the phage, its temperature-dependent cloning is obviously related to the presence of some lethal λ genes which are derepressed at 37°C. We have analyzed the structure of new plasmids which can be maintained at 37°C, and found that, although pSC101-f2 is not viable at this temperature the plasmids which survive are derived from it by DNA rearrangements.

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Abbreviations: CCC, covalently-closed circular form of DNA; Md, 10^6 daltons; OC, open circular form of DNA.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PATENTS

Examiner	:	J. Martinell
Group	:	1805
Applicant	:	Walter C. Fiers
Serial No.	:	387,503
Filed	:	July 28, 1989
For	:	DNA SEQUENCES, RECOMBINANT DNA MOLECULES AND PROCESSES FOR PRODUCING HUMAN FIBROBLAST INTERFERON-LIKE POLYPEPTIDES

Hon. Commissioner of Patents
and Trademarks
Washington, D.C. 20231

DECLARATION OF RICHARD L. CATE UNDER 37 C.F.R § 1.132

I, RICHARD L. CATE, declare:

1. I am generally familiar with the subject matter described and claimed in Fiers United States patent application 387,503 ("the Fiers application").

Considered 10/1/96

2. In May 1979 I received a Ph.D. in biochemistry from Kansas State University.

3. I have been working as a researcher in the field of molecular biology since June 1979. I worked in the laboratory of Dr. Walter Gilbert from June 1979 to October 1983. During that time I worked on the regulation of rat insulin genes. I also mapped some of the promoters on the cloning vector pBR322. I have worked at Biogen since November 1983.

During that time I have worked on the cloning of genes encoding factor VIII, lipocortin, and tumor necrosis factor, as well as being involved in a number of other projects, including being the project leader for cloning and expression of human Mullerian inhibiting substance. I have worked on research relating to IFN-ß since 1991.

4. I have read the July 29, 1994 Office Action.

5. I make this declaration to respond to certain of the Examiner's obviousness rejections under 35 U.S.C. § 103. In particular, I have considered the following documents that the Examiner has cited:

> Taniguchi et al., "The nucleotide sequence of human fibroblast interferon cDNA", Gene, 10, pp. 11-15 (1980) ("Taniguchi")(Ex. 1);
>
> Roberts et al., "Synthesis of simian virus 40 t antigen in Escherichia coli", Proc. Natl. Acad. Sci. USA, 76, pp. 5596-5600 (1979) ("Roberts")(Ex. 2).

6. I have also considered the Examiner's comments regarding the expression of IFN-α, which is referred to in the Fiers application and is reported in:

> Nagata et al., " Synthesis in E.coli of a polypeptide with human leukocyte interferon activity", Nature, 284, pp. 316-20 (1980) ("Nagata")(Ex. 3);

7. In 1980 several groups, having the DNA encoding IFN-ß in hand, were attempting to express that DNA to produce recombinant IFN-ß polypeptide. Among those groups were Dr. Fiers on behalf of Biogen, Dr. Goeddel's group at Genentech, and Dr. Taniguchi's group, at the Juridical Foundation in Japan.

As far as I am aware, Dr. Fiers was the first person in the world to successfully achieve expression of IFN-ß.

8. In 1980, when Dr. Fiers expressed IFN-ß, few proteins had been expressed by recombinant methods. Results were unpredictable -- there were both general and specific problems expected in the expression of recombinant proteins. The expression of one protein did not permit prediction of the successful expression of another protein having different chemical and physical properties.

9. In fact, it was not until the complete DNA sequence encoding IFN-ß was known that the specific problems in its expression became known. Dr. Taniguchi himself pointed out these problems when he reported that DNA sequence in Taniguchi. In fact, Taniguchi noted the following unique properties of IFN-ß based on the amino acid sequence deduced from the complete DNA sequence:

> (a) an abundance of hydrophobic amino acids, such as leucine (25), isoleucine (11), phenylalanine (9) and valine (5);
>
> (b) the presence of three Cys residues (positions 17, 31 and 141), consistent with the notion that S-S bonds may play a role in the maintenance of IFN-ß activity;
>
> (c) a codon preference for certain amino acids, including use of an infrequently used codon, AUA for 2 Ile residues;
>
> (d) the sequence for mature IFN-ß begins with the Met codon, unusual in eukaryotic proteins.

10. Based on these observations, I believe that the ordinary skilled worker attempting to express IFN-ß in 1980 would have had serious concerns about the expressibility of the IFN-ß DNA sequence, the hydrophobicity and correct folding and solubility of recombinant IFN-ß, its rapid proteolytic degradation, its bioinactivity when produced recombinantly, and its toxicity to the host. Particularly, I believe that the ordinarily skilled worker would have been concerned about the expressibility of IFN-ß given Nagata's very low level of expression of IFN-α -- one to two molecules per cell.

11. Each of these concerns was not hypothetical -- each concern was in fact a problem in the expression and recovery of biologically and immunologically active recombinant IFN-ß. Below I discuss why these concerns made the expression of IFN-ß unpredictable.

IFN-ß's Extreme Hydrophobicity

12. IFN-ß is surprisingly hydrophobic -- more so than SV 40 t antigen, and even more so than IFN-α. Numerous articles confirm this. See, e.g., Jankowski et al., "Binding Of Human Interferons To Immobilized Cibacron Blue F3GS: The Nature Of Molecular Interaction", Biochemistry, 15, pp. 5182-87, abstract (1976) (Ex. 4) ("All data point to a higher intrinsic hydrophobicity of human fibroblast interferon [IFN-ß] vis-a-vis human leukocyte interferon [IFN-α]"); Hayes et al., "Chou-Fasman

Analysis Of The Secondary Structure Of F And Le Interferons", Biochem. Biophys. Res. Comm., 95, pp. 872-79, 877 (1980) (Ex. 5) ("F [IFN-ß] interferon appears to be more hydrophobic than Le [IFN-α] interferon"); Fiers et al., "The Human Fibroblast And Human Immune Interferon Genes And Their Expression In Homologous And Heterologous Cells", Phil. Trans. R. Soc. Lond., B 299, pp. 29-38, 30 (1982) (Ex. 6) ("IFN-ß ... is much more hydrophobic [than] ... IFN-α"); Sulkowski et al., "Hydrophobic Properties Of Interferons", Ann. N.Y. Acad. Sci., 350, pp. 339-46, 344 (1980) (Ex. 7) ("a much more pronounced apparent hydrophobicity of HF-IF [IFN-ß than IFN-α]").

13. The Fiers application also confirms that IFN-ß is more hydrophobic than IFN-α. The Fiers specification indicates that IFN-ß added to an S-100 extract is recovered with only 10% efficiency, whereas IFN-α is recovered with 100% efficiency. See the Fiers application, p. 75, lines 16-31. The Fiers application then suggests that hydrophobicity may be the reason. See p. 77, lines 15-30.

14. Because of IFN-ß's hydrophobicity, the ordinarily skilled artisan would have had serious concerns that recombinantly produced IFN-ß would adhere to cellular membranes, and thus, preclude recovery of any active product.

15. IFN-ß does, in fact, adhere to membranes. See the Fiers application, p. 75, lines 16-31. See also, e.g., Fiers et al. "The Human Fibroblast And Human Immune Interferon

Genes And Their Expression In Homologous And Heterologous Cells", Phil. Trans. R. Soc. Lond., B, 299, pp. 29-38, 34 (1982) ("Fiers") ("Possibly the human IFN-ß, by its very hydrophobic nature, interacts with some essential bacterial component, like the inner side of the cell membrane."); Derynck et al., "Expression Of Human Fibroblast Interferon Gene In Escherichia coli", Nature, 287, pp. 193-97, 195 (1980) (Ex. 8) ("The observed decrease [in antiviral activity] is presumably due to non-specific sticking to the dialysis membranes, as HF-IF [IFN-ß] is known to be rather hydrophobic, and the unglycosylated bacterial form may be even more so.")

16. Nagata shows how important IFN-ß's hydrophobicity could be to the detection of expression of active recombinant product. Nagata obtained only about 1-2 molecules of active IFN-α per cell. See, p. 32, left column, second paragraph. This same level of expression would not have been detectable for IFN-ß -- the IFN-ß would have been adhered to the cell membrane. See, e.g., Rubinstein et al., "The Structure Of Human Interferons", Biochim. Biophys. Acta, 695, pp. 5-16, 12 (1982) ("Rubinstein") (Ex. 9) which states:

> "HuIFN-ß is less stable than HuIFN-α; its biological activity being significantly reduced by oxidation, adsorbance on walls at low protein concentrations or aggregation at high protein concentrations."

17. Because of the extreme hydrophobicity of IFN-ß, in my opinion, the ordinarily skilled artisan in 1980 would not

have had a reasonable expectation of success in attempts to express recombinant IFN-ß.

IFN-ß's Three Cys Residues

18. Because IFN-ß has 3 Cys residues, one of ordinary skill in the art in 1980 would be concerned with whether cytoplasmically produced IFN-ß would form the correct disulfide bonds so as to be biologically active.

19. I understand that the Examiner has stated that:

> "It is not necessary for one of ordinary skill in the art to know every detail of polypeptide folding ... or disulfide linkage .... At the time the instant invention was made, the main problems confronting scientists attempting to express foreign polypeptides were the transcription of the foreign polypeptide and the translation of the mRNA produced by transcription." (Office Action, p. 5).

20. I disagree with the Examiner. In my opinion, concerns with production of active recombinant IFN-ß polypeptide in 1980 would necessarily have encompassed post-translational events in addition to transcription and translation.

21. The Examiner's assertion is not correct for proteins synthesized as precursor proteins that must undergo proteolytic processing to be active. Two examples of such proteins are somatostatin and insulin. Both were expressed in E. coli under contrived conditions, by specifically taking advantage of information that was known about their post-translational processing. See e.g., Itakura et al., "Expression in Escherichia coli of a chemically synthesized gene for the

hormone somatostatin", Science, 198, pp. 1056-63 (1977) (Ex. 27); Goeddel et al., "Expression in Escherichia coli of chemically synthesized genes for human insulin", Proc. Natl. Acad. Sci USA, 76, pp. 106-10 (1979) (Ex. 28).

22. The characterization of IFN-ß that had been accomplished by 1980 could not rule out the possibility that IFN-ß was synthesized as a precursor protein that underwent post-translational processing during secretion from the cell. The molecular weight of IFN-ß was reported by most groups to be 20,000. See, e.g., Tan et al., "The Isolation And Amino Acid/Sugar Composition Of Human Fibroblastoid Interferon", Jour. Biol. Chem., 254, pp. 8067-73, 8069 (1979) (Ex. 11). It was also known that treatment with glycosidases, to remove the carbohydrate residues, resulted in a reduction in molecular weight of the native protein, with each polysaccharide chain having a molecular weight of 1,000 to 3,000. See, e.g., Bose et al., "Apparent dispensability of the carbohydrate moiety of human interferon for antiviral activity", J. Biol. Chem., 251, pp. 1659-62 (1976) (Ex. 26). For example, after deglycosylation, the molecular weight of IFN-α was reduced by about 4,000 (Ibid.). Upon cloning the human IFN-ß cDNA, it could be determined that the mature coding sequence predicted a protein with a molecular weight of 20,027. Since glycosylation would be expected to increase the molecular weight of the protein to about 24,000, a discrepancy existed between the

molecular weight predicted by the nucleotide sequence and the observed molecular weight of the native protein. One explanation for this discrepancy was that IFN-ß underwent processing prior to secretion. In fact, Dr. Michael Houghton suggested this possibility. See Houghton, "Human interferon gene sequences", Nature, 285, p. 536 (1980) (Ex. 19) ([A]t this stage, one cannot completely rule out the possibility of additional processing of F-IF [IFN-ß] besides the cleavage of the N-terminal signal sequence").

23. Further, post-translational processing in IFN-ß -- i.e., formation of the correct disulfide linkage -- is essential for proper folding and activity. In 1980, it was known that the exposure of native IFN-ß to reducing conditions resulted in inactivation and that, therefore, disulfide bridges were important for IFN-ß's biological activity. See, e.g., Vilcek et al., "Antigenic, Physicochemical, And Biologic Characterization Of Human Interferons", Ann. N.Y. Acad. Sci., 284, pp. 703-10, 705 (1977) ("Vilcek") (Ex. 10). Vilcek at p. 705, Table 2, reports that in the presence of reducing agents without SDS, IFN-ß was inactive. Dr. Taniguchi himself noted the importance of disulfide bridge formation. See Taniguchi, p. 14 ("There are three cystein residues (positions 17, 31, and 141) in human fibroblast interferon which is consistent with the notion that S-S bonds may play a role in the maintenance of the interferon activity."). See also Tan et al., "The Isolation And

Amino Acid/Sugar Composition Of Human Fibroblastoid Interferon", Jour. Biol. Chem., 254, pp. 8067-73, 8071 (1979).

24. Subsequent art also confirms this. See, e.g., Shepard et al., "A Single Amino Acid Change In IFN-β_1 Abolishes Its Antiviral Activity", Nature, 294, pp. 563-65, 564 (1981) (Ex. 12) ("[P]reincubation of IFN-β_1 extracts with dithiothreitol completely abolishes antiviral activity in a CPE inhibition assay ... it is likely that the disulphide bond between Cys 31 and Cys 141 is essential to maintain the structure of IFN-β_1 required for antiviral activity.")

25. Thus, in 1980, the ordinarily skilled artisan would have been greatly concerned with the correct folding of recombinant IFN-ß to produce an active polypeptide.

26. However, in 1980, there are several reasons why one of ordinary skill in the art could not have predicted that the "correct" disulfide bridge would form (or even that any disulfide bonds would form at all). And without that bridge, the produced protein, if any, would display no IFN-ß activity.

27. First, in 1980, the skilled artisan would not expect disulfide bridge formation in the cytoplasm because of the known reducing conditions there. Also, in certain hosts, specific enzymes were known to be involved in reduction of protein disulfides in the cytoplasm. For example, it was known that the E.coli thioredoxin system was involved in reduction of disulfide bridges to free sulfhydryl groups. See, e.g.,

Holmgren, "Reduction Of Disulfides By Thioredoxin", J. Biol. Chem., 254, pp. 9113-19 (1979) (Ex. 13).

28. Second, random interaction of the three Cys residues within recombinant IFN-ß protein could result in the formation of three possible intra-molecular disulfide bridges during cytoplasmic production or in vitro refolding. Yet only one would be "correct" and, thus, active. Intra-molecular refolding was, in fact, a problem. Mark et al., "Site-specific Mutagenesis Of The Human Fibroblast Interferon Gene", Proc. Natl. Acad. Sci. USA, 81, pp. 5662-66 (1984) ("Mark") (Ex. 14) reported that E.coli-produced recombinant IFN-ß did not form the correct intra-molecular disulfide bridge -- and, when refolded in vitro, "preferentially form[ed] the wrong disulfide bridges." See Mark, p. 5666 (emphasis added).

29. Third, one or more of the Cys residues, and particularly the "free" third Cys residue, could be involved in inter-molecular disulfide bridging, resulting in the formation of inactive dimers or oligomers. This, too, was a problem. Mark also found that when refolded in vitro, most of the recombinantly produced IFN-ß "existed as dimers and oligomers" which were inactive. See, Mark, p. 5662 (emphasis added).

IFN-ß's Surprising Codon Usage

30. Taniguchi also noted a surprising codon usage in IFN-ß. Among this codon usage was the infrequently used AUA

codon for Ile. If the expression system did not have sufficient quantities of tRNA to recognise and translate these unusual codons, expression would be affected.

IFN-ß's Initiating AUG

31. Taniguchi also noted that mature IFN-ß began with a Met. This is unusual. The majority of eukaryotic proteins expressed prior to 1980 did not begin with Met.

General Problems With IFN-ß Expression

32. In addition to the specific problems expected in the expression of IFN-ß, there were a number of general concerns that precluded the ordinarily skilled artisan from reasonably expecting that active recombinant IFN-ß would be successfully expressed in 1980. These included concerns with proteolytic degradation, bioinactivity, toxicity and solubility of recombinant IFN-ß.

33. Rapid proteolytic degradation and bioinactivity were, in fact, encountered. When Dr. Taniguchi expressed IFN-ß (subsequent to Dr. Fiers), he reported that recombinant pre-IFN-ß was completely degraded in 50 minutes, and mature IFN-ß was 50% degraded in 50 minutes. He also reported that extracts of pre-IFN-ß were totally inactive and the level of mature IFN-ß antiviral activity was only about 1% of estimated IFN-ß synthesis. See Taniguchi, "Expression Of The Human

Fibroblast Interferon Gene In Escherichia coli", Proc. Natl. Acad. Sci. USA, 77, pp. 5230-33 (1980) ("Taniguchi II") (Ex. 15).

34. In addition, problems with IFN-ß's toxicity were also encountered -- synthesis of IFN-ß in bacteria was severely growth inhibitory, although accounting for only 2% of total cellular protein. See Remaut et al., "Inducible High Level Synthesis Of Mature Human Fibroblast Interferon In Escherichia coli", Nucl. Acids Res., 11, pp. 4677-88 (1983) (Ex. 16).

35. Finally, problems with the solubility of recombinant IFN-ß were encountered. Mark, supra, at p. 5664, found that most of the recombinant IFN-ß was produced in insoluble, biologically inactive form.

IFN-ß and IFN-α Are Different Proteins

36. I understand that the Examiner has noted that Nagata's expression of IFN-α was carried out before "the priority date of the instant application" and has referred to p. 9 of the Fiers application.

37. As I stated above, in 1980 the expression of one recombinant protein did not permit prediction of the successful expression of another protein having different chemical and physical properties. IFN-ß and IFN-α are distinct proteins having distinct properties. Taniguchi, p. 14, notes this, stating "fibroblast and leukocyte interferons exhibit a number

of different properties". Further, Vilcek, p. 708-09 states: "[t]he implication of these findings is that the structural differences existing between Le [IFN-α] and F [IFN-β] interferons do influence some characteristics of their biological activities."

38. Fiers, supra, at p. 30, identifies three differences in the chemical properties of IFN-α and IFN-β: "IFN-β is more labile, is much more hydrophobic and it is a glycoprotein whereas IFN-α is not."

39. First, as I outlined above, IFN-β is much more hydrophobic than IFN-α. The hydrophobicity of IFN-β causes it to adhere to cell membranes. As the Fiers application, p. 75, lines 16-31, reports, only 10% recovery of IFN-β from cell extracts was possible. In contrast, the Fiers application reports 100% recovery of IFN-α from cell extracts. Id. See also Adam et al., Vnitr Lek., 40, pp. 329-33 (1994) (Ex. 17: English abstract) ("Interferon beta differs from interferon alpha by a higher lipophilia and thus greater tissue affinity.") Because of IFN-β's extreme hydrophobicity, the ordinarily skilled artisan would not have expected to detect any active IFN-β at the same level of expression reported by Nagata. Supra, ¶¶ 10, 16.

40. The secondary structure of IFN-β differs significantly from IFN-α, perhaps explaining its greater hydrophobicity. See, e.g., Hayes, "Chou-Fasman Analysis Of The

Secondary Structure Of F And Le Interferons", Biochem. Biophys. Res. Comm.,95, pp. 872-79, 877 (1980), which states:

> "F [IFN-ß] interferon contains an approximately equal amount of ß pleated sheet and α helical residues, while Le [IFN-α] interferon has significantly more α helix ... Since β pleated sheets are usually composed of hydrophobic residues and tend to associate with one another via hydrophobic interactions, perhaps this explains the experimental observation that F interferon appears to be more hydrophobic than Le interferon."

41. IFN-ß is also less stable than IFN-α. This too was a concern in the expression of an active molecule. IFN-ß's instability is confirmed in, e.g., Rubinstein, supra, at p. 12 ("HuIFN-ß is less stable than HuIFN-α").

42. Concerns with IFN-ß's instability were compounded by the knowledge in 1980 that deglycosylation of native IFN-ß reduced its stability. See Havell et al., "Suppression of human interferon production by inhibitors of glycosylation", Virology, 63, pp. 475-83 (1975) (Ex. 29). Thus, the skilled artisan would have been concerned that E.coli-produced recombinant IFN-ß would be even less stable than native IFN-ß due to the absence of glycosylation.

43. It was also known in 1980 that treatment with glycosylation inhibitors resulted in reduced yield of active IFN-ß. See Havell et al., p. 482, Ex. 29. The ordinarily skilled worker would have been concerned that absence of glycosylation in E.coli-produced IFN-ß would cause similar reductions in yield of recombinant IFN-ß. This would compound

concerns with the ability to detect any active IFN-ß, especially given the concerns based on Nagata's low yield and IFN-ß's extreme hydrophobicity (supra, ¶¶ 10, 12-16).

44. Further, while Nagata's IFN-α was produced in soluble, biologically active form in the cytoplasm, the major portion of IFN-ß is produced in insoluble biologically inactive form. See Mark, p. 5664.

45. In addition, IFN-ß and IFN-α are translated from distinct mRNAs. See Taniguchi et al., "Human Leukocyte And Fibroblast Interferons Are Structurally Related", Nature, 285, pp. 547-49, 547 (1980) (Ex. 18). Antibodies to IFN-ß do not neutralize IFN-α, and vice-versa. Ibid. The two proteins have distinct amino acid sequences, having only 29% homology. And there is only a 45% homology between their nucleotide sequences, with the longest common stretch being only 13 nucleotides. See Houghton et al., "Human Interferon Gene Sequences", Nature, 285, p. 536 (1980) (Ex. 19).

46. For all these reasons, in my opinion, Nagata's expression of IFN-α did not provide the ordinarily skilled artisan with a reasonable expectation of success in the expression of IFN-ß. I believe that this is best proved by the actions of others at that time.

47. In fact, none of the groups who succeeded in expressing IFN-ß -- applicant, and subsequently Dr. Taniguchi and Dr. Goeddel -- used the Nagata method. Dr. Taniguchi's

subsequent expression is reported in Taniguchi II, supra (September 1980). Dr. Goeddel's subsequent expression is reported in Goeddel et al., "Synthesis Of Human Fibroblast Interferon By E.coli", Nucl. Acids Res., 8, pp. 4057-74 (August 1980) ("Goeddel") (Ex. 20).

Roberts Does Not Make Expression Of IFN-ß Obvious

48. I understand that the Examiner has rejected the claims of the Fiers application as obvious over the DNA encoding IFN-ß in view of Roberts. In his rejection, the Examiner states that transcription and translation were the main problems confronting scientists in 1980, and that Roberts "solves" these problems. I also understand that the Examiner contends that the differences between SV 40 t antigen and IFN-ß are not relevant.

49. I agree with the Examiner that achieving transcription and translation of recombinant proteins were significant problems in 1980. I do not, however, believe that Roberts can or does solve all the transcriptional and translational problems for all recombinant proteins.

50. Further, in 1980, as I stated above, I believe the ordinarily skilled artisan in 1980 would have been concerned with post-translational concerns in attempts to express IFN-ß. Because correct folding was essential to IFN-ß's activity, the skilled artisan would have been concerned with post-translational problems -- such as correct disulfide bridge

formation -- that might affect folding, and thus activity. Supra, ¶¶ 23-29. They would also have had concerns about other properties of the product, if transcribed and translated, that would preclude recovery of any active IFN-ß. Supra, ¶¶ 32-35. Thus, there were concerns about IFN-ß's hydrophobicity, bioinactivity, toxicity, solubility, as well as concerns about proteolytic degradation.

51. The Examiner goes on to state that "[a]pplicant has provided no convincing evidence that one of ordinary skill in the art would have been prevented from using the Roberts et al method in the expression of IFN-ß."

52. In my opinion the Examiner is looking at the situation backwards. There would have been nothing preventing the ordinarily skilled artisan from trying a number of expression systems, including Roberts, in attempts to express IFN-ß. However, back in 1980, when faced with the problem of IFN-ß expression, there would be no reason for the ordinary skilled artisan to select Roberts over any of a number of other publications in attempts to express recombinant IFN-ß. And the ordinarily skilled artisan would not have had a reasonable expectation of success with any of those methods, because of the unique physical and chemical properties of IFN-ß.

53. In fact, in my opinion, there would be good reasons not to select Roberts. Roberts describes the expression of SV 40 t antigen. Because SV 40 t antigen is so physically

and chemically dissimilar to IFN-ß, the ordinarily skilled artisan would not have chosen the Roberts method to attempt expression of IFN-ß.

54. I understand that the Examiner has referred to a statement in Roberts, at p. 5600, that the method described there has "general use in the expression of eukaryotic genes."

55. I do not agree with the Examiner. Roberts does not say it is of general use. Instead Roberts merely says that it provides "a rational approach to the problem of obtaining expression of eukaryotic genes in bacteria". I do not believe that this statement establishes that the Roberts method is of general use. To the contrary, it acknowledges that heterologous gene expression was a "problem". All Roberts provides is an "approach".

56. Many early gene expression articles contained similar statements predicting that the reported approach "had potential" general applicability. See, e.g., Villa-Komaroff et al., "A Bacterial Clone Synthesizing Proinsulin," Proc. Natl. Acad. Sci. USA, 75, 8, pp. 3727-31 (1978) (Ex. 21) ("[c]learly we have exploited a general method that should lead to the expression and secretion of any eukaryotic protein provided another protein, such as penicillinase, will serve as a carrier, by virtue of its leader sequence"); Seeburg et al., "Synthesis Of Growth Hormone By Bacteria," Nature, 276, pp. 795-98 (1978) (Ex. 22) ("Our findings and those of Villa-Komaroff et al.

indicate that coding sequences from higher organisms can be expressed in bacteria. Thus, it should be possible to produce biologically important peptides with the use of recombinant DNA techniques and naturally occurring structural gene sequences").

57. For this reason too, in my opinion, the statement that the Examiner has pointed to does not provide any basis for selecting the Roberts approach.

58. As I stated above, the ordinarily skilled artisan would have had good reasons not to select Roberts. There is nothing in Roberts that motivates the skilled artisan to select that "general" approach over any other as a solution to the specific problems expected in the expression of IFN-ß. Roberts does not even mention IFN-ß.

59. Unlike IFN-ß, SV 40 t antigen is a cytoplasmic protein. It is unglycosylated, slightly basic protein of 174 amino acids.

60. Because it is a cytoplasmic protein, SV 40 t antigen would be expected to be soluble, correctly folded and active in such a reducing environment. This was not the expectation for IFN-ß. Thus, Roberts expression of SV 40 t antigen could not provide any guidance in solving these specific concerns with the expression of recombinant IFN-ß. Nor does Roberts provide any guidance for solving the problems expected with rapid proteolytic degradation and toxicity of IFN-ß to the host.

61. For these reasons, in my opinion, the ordinarily skilled artisan having the sequence of IFN-ß in hand, would not have selected Roberts to express that sequence. And if they had selected Roberts, the ordinarily skilled artisan would have had no reasonable expectation of success in producing active recombinant IFN-ß.

62. The contemporaneous actions of the workers in the field is good evidence of this. Neither Dr. Fiers, nor any of the other groups (Dr. Taniguchi and Dr. Goeddel) that subsequently expressed IFN-ß, selected the Roberts approach. See Taniguchi II and Goeddel. Most importantly, Dr. Roberts and Dr. Ptashne, who knew the most about Roberts, did not select that approach when they expressed IFN-ß in collaboration with Dr. Taniguchi. See Taniguchi II, supra. Instead, they selected a new method, referred to in Guarente et al., "Improved methods For Maximizing Expression Of A Cloned Gene: A Bacterium That Synthesizes Rabbit ß-Globin", Cell, 20, pp. 543-53 (1980) ("Guarente") (Ex. 23), which I am informed and believe was not published until after June 6, 1980. Guarente itself characterised the Roberts approach as having an "important limitation" in requiring the use of a functional gene product assay because identification of expressing clones may be "laborious or impossible". See Guarente, p. 544.

63. The contemporaneous actions of others are also good proof of what workers in the art thought was a patentable

advance. Dr. Fiers was not the only one who thought expression of IFN-ß was patentable. Both Dr. Goeddel and Dr. Taniguchi also thought the same thing. I am informed and believe that they also filed patent applications directed to the expression of IFN-ß. See published European patent applications EP-A 0 048 970 (Goeddel) (Ex. 24) and EP-A2 0 042 246 (Taniguchi) (Ex. 25).

64. For these reasons too, in my opinion, Dr. Fiers expression of IFN-ß for the first time, was not obvious in view of the other recombinant proteins expressed in 1980.

Conclusion

65. For the foregoing reasons, in my opinion, the ordinarily skilled artisan in 1980 would not have had a reasonable expectation of success in attempts to express recombinant IFN-ß.

66. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true. I further declare that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the subject application or any patent issuing thereon.

Richard L. Cate

Richard L. Cate

Signed at Cambridge, Massachusetts
this 20 day of January, 1995.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PATENTS

Examiner	:	Not Yet Assigned
Group	:	Not Yet Assigned
Applicant	:	Walter C. Fiers
Filed	:	June 6, 1995
Serial No.	:	08/471,646
For	:	DNA SEQUENCES, RECOMBINANT DNA MOLECULES AND PROCESSES FOR PRODUCING HUMAN FIBROBLAST INTERFERON-LIKE POLYPEPTIDES

Hon. Assistant Commissioner for Patents
Washington, D.C. 20231

SUPPLEMENTAL DECLARATION OF
RICHARD L. CATE UNDER 37 C.F.R § 1.132

I, RICHARD L. CATE, declare:

Considered 10/11/96

1. I am generally familiar with the subject matter described and claimed in Fiers United States patent application 387,503 ("the Fiers application"). I am also familiar with the Examiner's July 29, 1994 Office Action in United States patent application SN 387,503, filed July 28, 1989. Part of the response to that Office Action included my earlier declaration, the Declaration of Richard L. Cate Under 37 C.F.R. § 1.132 ("Cate Decl. __"), filed January 29, 1995.

2. I have read the Examiner's March 9, 1995 Advisory Action in the parent application. I make this supplemental declaration to respond to certain of the Examiner's comments in

the Advisory Action and to clarify the statements made in my January declaration.

EXPRESSION OF RECOMBINANT IFN-ß WAS UNPREDICTABLE

3. In the Advisory Action, the Examiner states that:

> "declarant's opinion that one of ordinary skill in the art would have some 'concern' that the method outlined in the rejection would not work is not convincing because success need not be guaranteed."

4. I believe that the Examiner has misunderstood various statements in my January declaration. In my January declaration, I explained in detail the concerns and problems expected in the recombinant production of IFN-ß. Cate Decl. ¶¶ 9-17, 22-35, 39-44. I then stated my opinion that because of these concerns one of ordinary skill in 1980 would not and did not have a reasonable expectation of success in attempts to express recombinant IFN-ß. Cate Dec. ¶¶ 17, 32, 46, 52, 61, 65. I also stated my opinion that the expression of recombinant IFN-ß at that time was "unpredictable." Cate Decl. ¶ 11.

THE PRIOR ART IDENTIFIED (BUT DID NOT SOLVE) THE PROBLEMS EXPECTED IN THE RECOMBINANT PRODUCTION OF IFN-β

5. In his Advisory Action, the Examiner also stated:

> "the [Cate] declaration and argument rely on evidence found subsequent to the filing date to show that those of ordinary skill in the art might have concern over whether the method outlined in the rejection would work, but fail to explain how one of ordinary skill in the art could be deterred or could even take into consideration information that was unknown at the time the invention was made."

The Examiner goes on to say that documents "published after the filing date of the present application can have no weight at all." Advisory Action, p. 2.

6. I do not agree with the Examiner. The problems expected in the recombinant production of IFN-β were known on June 6, 1980. I have called the subsequent articles to the Examiner's attention only to show that the expected problems turned out to be real and significant problems.

A. General Problems

7. Based on the prior art expression of other recombinant proteins, in June 1980 the skilled artisan would have expected a number of general problems in the production of any recombinant protein. These included rapid proteolytic degradation, bioinactivity, insolubility and toxicity of the recombinant protein in the transformed host. See Cate

Decl. ¶¶ 32-35. These problems had all been encountered before June 6, 1980 with other recombinant proteins, as shown below:

Proteolytic degradation/Bioinactivity

Bassford et al., "Use of Gene Fusion To Study Secretion of Maltose-Binding Protein Into Escherichia coli Periplasm", J. Bacteriol., 139, pp. 19-31, 29-30 (1979) ("Bassford") (Ex. 1);
Itakura et al., "Expression In Escherichia coli Of A Chemically Synthesized Gene For The Hormone Somatostatin", Science, 198, pp. 1056-63, 1060 (1977) ("Itakura") (Ex. 2);
Goeddel et al., "Direct Expression In Escherichia coli of A DNA Sequence Coding For Human Growth Hormone", Nature, 281, pp. 544-48, 548 (1979) ("Goeddel 2") (Ex. 3);
Fraser and Bruce, "Chicken Ovalbumin Is Synthesized And Secreted By Escherichia coli," Proc. Natl. Acad. Sci USA, 75, pp. 5936-40, 5939 (1978) (Ex. 4).

Toxicity

Bassford, supra;
Emr et al., "Mutation Altering The Cellular Localization Of The Phage Lambda Receptor, An Escherichia coli Outer Membrane Protein", Proc. Natl. Acad. Sci. USA, 75, pp. 5802-06, 5803 (1978) (Ex. 5).

Insolubility

Itakura, supra.

The same general problems were to be expected in the recombinant production of IFN-ß. However, the extent to which these expected problems would, in fact, be problems in the recombinant production of IFN-ß, was simply not predictable.

8. In my January declaration I referred to Taniguchi, "Expression Of The Human Fibroblast Interferon Gene in Escherichia coli", Proc. Natl. Acad. Sci. USA, 77, pp. 5230-

33 (1980) ("Taniguchi II") (Ex. 6), Mark et al., "Site-specific Mutagenesis Of The Human Fibroblast Interferon Gene", Proc. Natl. Acad. Sci. USA, 81, pp. 5662-66 (1984) ("Mark") (Ex. 7), and Remaut et al., "Inducible High Level Synthesis Of Mature Human Fibroblast Interferon in Escherichia coli", Nucl. Acids Res., 11, pp. 4677-88 (1983) (Ex. 8). These papers are dated after the Fiers' application. They demonstrate that the problems expected (i.e., proteolytic degradation, bioinactivity, toxicity, and insolubility) in 1980 did, in fact, turn out to be problems. They also demonstrate that the expected problems were very significant ones. It is for these reasons that the post-published papers are informative.

However, to be very clear, the skilled artisan would have expected the general problems mentioned above on the basis of papers published by June 6, 1980. There is no information in the post-published papers that the skilled artisan would have required to expect to encounter those problems on June 6, 1980. The subsequent papers only confirm what one would have expected. There were problems, and very significant ones at that.

B. Specific Problems

9. Specific problems with the expression of recombinant IFN-ß were also expected by the skilled workers on June 6, 1980. These problems were also identified in and well recognized from papers published before June 6, 1980. I cited

several of those papers in my January declaration. I also cited several articles dated after June 6, 1980 to demonstrate that the specific problems that one of skill in the art would have expected in 1980 also were, in fact, problems -- and significant ones in expressing IFN-ß.

IFN-ß's Hydrophobicity

10. For example, IFN-ß's hydrophobicity was known by June 6, 1980. In fact, Dr. Taniguchi noted IFN-ß's hydrophobicity. See, e.g., Taniguchi et al., "The nucleotide sequence of human fibroblast interferon cDNA", Gene, 10, pp. 11-15 (1980) (Ex. 9). Cate Decl. ¶ 9.

Others also reported that IFN-ß was extremely hydrophobic -- more so even than IFN-α. See, e.g., Jankowski et al., "Binding Of Human Interferons To Immobilized Cibacron Blue F3GS: The Nature Of Molecular Interaction", Biochemistry, 15, pp. 5182-87, abstract (1976) ("All data point to a higher intrinsic hydrophobicity of human fibroblast interferon [IFN-ß] vis-a-vis human leukocyte interferon [IFN-α]") (Ex. 10). Fantes, "Interferons: Chemical Properties", Tex. Reports. Bio. Med., 35, pp. 173-80 (1977) ("Fantes") ("Fibroblast interferon is much more hydrophobic than leucocyte interferon"). (Ex. 20). Cate Decl. ¶ 12.

11. As I set out in my January declaration, because of IFN-ß's extreme hydrophobicity, the ordinarily skilled

artisan had no reasonable expectation of success in the recombinant production of IFN-β because the skilled worker would have had serious concerns that recombinantly produced IFN-β would adhere to cellular membranes and other surfaces, and preclude recovery of any active product. Cate Decl., ¶¶ 14-17. Particularly, this would have been a concern for IFN-β expression in view of the 1-2 molecules per cell expression reported by Nagata, Nature, 284, pp. 316-20 (1980) for the much less hydrophobic IFN-α.

12. The tendency of IFN-β to adhere to surfaces was well known in 1980. In fact, researchers working with native IFN-β in vitro used cytochrome c to reduce or prevent such adherence. See, e.g., Anfinsen et al., "Partial purification of human interferon by affinity chromatography", Proc. Natl. Acad. Sci. USA, 71, pp. 3139-42, 3141 (1974) (Ex. 11); Sedmak and Grossberg, "Stabilization of interferons", Texas Reports Biol. Med., 35, pp. 198-203, 200 (1977) ("Sedmak and Grossberg") (Ex. 12). Such in vitro use of cytochrome c to reduce the recognized problem of adherence could not be implemented in recombinant expression of IFN-β where problems with intracellular adherence were expected.

13. Knight, "Interferon: Purification and initial characterization from human diploid cells", Proc. Natl. Acad. Sci. USA, 73, pp. 520-23, 522 (1976) (Ex. 13) also reports that purified IFN-β was unstable in dilute solutions, losing 50-75%

of its activity within 24 hours. Knight attributed the loss to adsorption of the IFN-ß to "surfaces". Knight, p. 522.

14. The Fiers application confirms that IFN-ß does, in fact, adhere to membranes. See p. 75, lines 16-31.

15. As I stated in my January declaration, these concerns were compounded in June 1980 by the knowledge that E.coli-produced IFN-ß would be unglycosylated. This is because it was known that production of native IFN-ß in the presence of inhibitors of glycosylation decreased its stability even further, and substantially reduced its yield. Cate Decl. ¶¶ 42-43. See also, Knight, p. 522.

16. For these reasons, in June 1980 because of IFN-ß's extreme hydrophobicity, the ordinarily skilled artisan had no reasonable expectation of success in the recombinant production of active IFN-ß.

IFN-ß's Three Cys Residues

17. By June 6, 1980, it was also known that IFN-ß has three Cys residues. Taniguchi notes this. Because of its three Cys residues, one of ordinary skill in the art in 1980 would have had no reasonable expectation of success in the recombinant production of IFN-ß because of serious concerns that cytoplasmically produced IFN-ß would not form the correct disulfide bonds that were known to be essential for biological activity.

18. As I set out in my January declaration, by June 6, 1980, there was no expectation that any disulfide bridges would form at all in the cytoplasmic reducing environment. Cate Decl. ¶ 27.

19. This is because it was known in June 1980 that, in certain hosts, specific enzymes were known to be involved in reduction of protein disulfides in the cytoplasm. For example, it was known that the E.coli thioredoxin system was involved in reduction of disulfide bridges to free sulfhydryl groups. See, e.g., Holmgren, "Reduction Of Disulfides By Thioredoxin", J.Biol. Chem., 254, pp. 9113-19 (1979) (Ex. 14). Cate Decl. ¶ 27.

It was also known by June 6, 1980, that the formation of disulfide bridges was essential for IFN-β's biological activity. Cate Decl. ¶ 23. See, e.g., Vilcek et al., "Antigenic, Physicochemical, And Biologic Characterization Of Human Interferons", Ann. N.Y. Acad. Sci., 284, pp. 703-10, 705 (1977) ("Vilcek") (Ex. 15). Vilcek at p. 705, Table 2, reports that exposure of native IFN-β to reducing conditions resulted in inactivation.

Dr. Taniguchi himself noted the importance of disulfide bridge formation. See Taniguchi, p. 14 ("There are three cystein residues (positions 17, 31, and 141) in human fibroblast interferon which is consistent with the notion that S-S bonds may play a role in the maintenance of the interferon

activity."). See also Tan et al., "The Isolation And Amino Acid/Sugar Composition Of Human Fibroblastoid Interferon", Jour. Biol. Chem., 254, pp. 8067-73, 8071 (1979) (Ex. 16).

20. Further, the ordinarily skilled artisan could not have predicted correct folding or formation of the "correct" disulfide bridge in a heterologous host. This is because three intra-molecular disulfide bridges were possible. Cate Decl. ¶ 28. In addition, because of the "free" third Cys residue, the formation of inter-molecular disulfide bridges was also possible, leading to formation of dimers or oligomers. Cate Decl. ¶ 29.

21. The concern that IFN-ß would form aggregates or disulfide mismatches arose from observations of native IFN-ß's instability during processing. When exposed to mechanical stress during processing, IFN-ß did in fact form random intermolecular disulphide bonds, and was substantially inactivated. See, e.g., De Somer et al., "Mass production of human in diploid cells", In The Production And Use Of Interferon For The Treatment And Prevention Of Human Virus Infections, The Tissue Culture Association, Rockville, MD., pp. 39-46 (1974) (99% loss of activity when subjected to rotational agitation) (Ex. 17); Edy et al., "Stabilization of mouse and human interferons by acid pH against inactivation due to shaking and guanidine hydrochloride", Proc. Soc. Exp. Biol. Med., 146, pp. 249-53 (1974) (99% loss of activity when subjected to end-

over-end agitation) (Ex. 18). In contrast, IFN-α remained active under these conditions. See, e.g, Sedmak and Grossberg, supra, p. 200.

22. In fact, researchers noted that reagents that inhibited intermolecular disulphide bond formation but that did not disrupt intramolecular disulphide bond formation were useful in stabilizing native IFN-ß in vitro. See, e.g., Sedmak and Grossberg, p. 201:

> "Cartwright, Senussi and Grady found that reagents which inhibit disulphide bond formation stabilized fibroblast interferon in the shearing test; their observations suggest that the shearing inactivation is a result of formation of intermolecular disulphide bonds which reducing agents prevent. They found that weakly reducing DL-thioctic acid was more effective than the strongly reducing dithiothreitol in stabilizing the interferon. They suggest that the thioctic acid may be more effective than dithiothreitol since thioctic acid only reduces the readily accessible intermolecular bonds, whereas dithiothreitol, of smaller molecular weight, may also reduce intramolecular disulphide bonds."

See also Fantes, p. 177 ("The stability of native fibroblast interferon to mechanical stress can be greatly increased by agents (e.g., thioctic acid, dithiothreitol, p-chloromercuribenzoate, etc.) that keep sulphydryl groups in a reduced state, without however reducing disulphide bonds"). Use of such in vitro additives in attempts to alleviate this recognized problem could not be implemented in the recombinant expression of IFN-ß.

23. And, as I stated in my January declaration, incorrect folding and dimer formation were, in fact, problems during recombinant production of IFN-ß. Cate Decl., ¶¶ 28-29.

24. To avoid the problem of the free (i.e., non-bridged) cys residues forming either covalent aggregates or mismatched disulfides, IFN-ß muteins have been developed lacking the third cys residue. See United States patent 4,752,585 (Ex. 19).

THE SKILLED ARTISAN WOULD NOT HAVE SELECTED THE ROBERTS APPROACH

25. Finally, in his Advisory Action, the Examiner stated:

> "[A]pplicant's assertion that the examiner 'has it backwards' in connection with applicant's failure to make a convincing argument that one of ordinary skill in the art would have been prevented from using the method of Roberts et al is most unconvincing." Advisory Action, pp. 2-3.

26. I believe that the Examiner is referring to ¶ 52 of my January declaration. As I understand it, the Examiner is assuming that the artisan would select the Roberts approach and, from that starting point, the Examiner contends that he is unpersuaded that the ordinarily skilled artisan would have then been prevented from using that approach. I respectfully disagree with the Examiner, for two reasons.

27. First, I do not agree with the Examiner's starting point. I do not believe that the ordinarily skilled

artisan would have selected the Roberts approach. In fact, as I stated in my January declaration, I believe the opposite -- in my opinion the ordinarily skilled artisan simply would not have been led to the Roberts approach. This is because Roberts does not mention IFN-ß. Roberts refers to expression of a dissimilar protein (i.e., SV 40 t antigen). The Roberts approach does not address or solve the specific problems expected in the expression of IFN-ß. Cate Decl. ¶ 52. To the contrary, Roberts expressly acknowledges that even heterologous gene expression in general was a "problem".

In 1980, there were, in fact, several approaches that might have been tried to express IFN-ß. There was no reason to select the Roberts approach from these. Cate Decl. ¶ 52. None gave a reasonable expectation of success in the recombinant production of IFN-ß. Cate Decl. ¶ 52.

28. Second, in my January declaration I outlined the reasons why the ordinarily skilled artisan would not have had a reasonable expectation of success in attempts to express recombinant IFN-ß using the Roberts approach. Cate Decl., ¶¶ 53-63. I have summarized these above in ¶¶ 7-23. Thus, to use the Examiner's language, there are good reasons why the ordinarily skilled artisan would have been deterred from using the Roberts approach.

29. For the foregoing reasons, in my opinion, the ordinarily skilled artisan on June 6, 1980 had no reasonable expectation of success in attempts to express recombinant IFN-ß.

30. I hereby declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true. I further declare that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. § 1001, and that such willful false statements may jeopardize the validity of the subject application or any patent issuing thereon.

Richard L. Cate

Signed at Cambridge, Massachusetts
this 2 day of August, 1995.

THIS OPINION WAS NOT WRITTEN FOR PUBLICATION

The opinion in support of the decision being entered today (1) was not written for publication in a law journal and (2) is not binding precedent of the Board.

Paper No. 265

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

DAVID V. GOEDDEL and SIDNEY PESTKA
Junior Party[1]

v.

CHARLES WEISSMAN
Senior Party[2]

Interference No. 101,601

FINAL HEARING: March 2, 1992

Before RONALD H. SMITH, DOWNEY and WILLIAM F. SMITH[3], Administrative Patent Judges.

DOWNEY, Administrative Patent Judge.

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[1] Application 06/256,204, filed April 21, 1981. Accorded the benefit of Application 06/164,986, filed July 1, 1980, application 06/205,578, filed November 10, 1980, application 06/184,909, filed September 8, 1980. Assignor to Genentech, Inc. and Hoffmann-LaRoche Inc.

[2] Application 06/471,301, filed March 2, 1983. Accorded the benefit of Application 06/223,108, filed January 7, 1981, European Patent Application 80301100.6, filed April 3, 1980, United Kingdom Patent Application 8031737, filed October 2, 1980. Assignor to Biogen N.V.

[3] APJ William F. Smith has been substituted for APJ Goolkasian who has retired. Cf. In re Bose Corp., 772 F.2d 866, 868-869, 227 USPQ 1, 2-4 (Fed. Cir. 1985).

cells to viral infection. The subject matter at issue is defined by a single count, which count is identical to claim 1 of the Goeddel et al. application. The count reads as follows:

COUNT 1

> A polypeptide of about 165-166 amino acids comprising the amino acid sequence of a mature human leukocyte interferon microbially produced and unaccompanied by any corresponding presequence or portion thereof.

Goeddel et al. claims 1-4, 8 and 52-69 and Weissmann claims 37-39, 41, 42, 77-79 and 85 and 87 correspond to the count.

During the preliminary motion stage of this proceeding, the parties filed numerous motions. However, only those motions raised by the parties in their briefs are dealt with herein.

The Examiner-in-Chief, hereinafter APJ[6], granted Weissmann motions (II.E.1, II.F and II.E.4) which added claims 77-79, 85 and 87 to his application to correspond to count 1 and which required Goeddel et al. to add claims 28, 30, 46-48, 50, 65, 67, 73, 75-78, 80-82, 85 and 86 to their application to

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[6] The Commissioner of Patents and Trademarks authorized Examiners-in-Chief to use the title Administrative Patent Judge (APJ) for business-related activities. See Commissioner's Notice of Oct. 15, 1993, New Title for Examiners-in-Chief, 1156 Off. Gaz. Patent & Trademark Office 32 (Nov. 9, 1993).

correspond to count 1. On November 7, 1988, Goeddel et al. added the noted claims to their application as claims 52-69.[7]

The APJ dismissed Goeddel et al. motions: (I.D.1) to add counts 2-4 to this interference and to be accorded benefit with respect thereto; (I.D.2) to add claims 28-40 to their application; and (I.D.3) to require Weissmann to add proposed claims "B" through "N" to his application. This decision was affirmed on reconsideration.

The APJ denied Goeddel et al. motions: (I.A.1 and I.A.2) to designate Weissmann claims 37-39, 41 and 42 and Goeddel et al. claims 4 and 8 as not corresponding to the count; (I.C.) for judgment on the grounds that Weissmann's claims 37-39, 41 and 42 are unpatentable to him under 35 USC 103; and (I.E.1 and I.E.2) to attack the benefit accorded Weissmann in the declaration notice of EPO application No. 80301100.6 filed April 3, 1980 and UK application No. 8031737, filed October 2, 1980. The APJ also denied Weissmann motion (II.C 1) to add counts 3-7 to the interference.

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[7] The interference is being concurrently redeclared to reflect the granting of these motions.

After the Weissmann testimony period closed, Goeddel et al. filed a second set of belated motions[8] attacking benefit accorded Weissmann of the April 3, 1980 EPO and October 2,1980 UK applications for failure to disclose the best mode (Paper Nos. 199-201). Opposition papers were filed (Paper Nos. 211-212, accompanied by Exhibit 213) as were replies (Paper Nos. 222-223). These papers were deferred to final hearing.

Weissmann filed a belated motion (37 CFR §1.628) to amend paragraph 14 of his October 21, 1987 preliminary statement (Paper Nos. 170 and 171). An opposition and reply were filed (Paper Nos. 174 and 182, respectively).

Various motions to strike or suppress were filed by the parties.

The main briefs of the parties raise the following issues:

1. Is Goeddel et al. entitled to raise at final hearing the dispositions of I.D.1, I.D.2, I.D.3, II.E.1, and II.F, and if so, did the APJ properly dismiss I.D.1, I.D.2, I.D.3 and properly grant-in-part II.E.1, and II.F?

2. Goeddel et al. case for priority of invention.

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[8] After the decision on preliminary motions had been rendered, Goeddel et al. filed a belated motion (37 CFR §1.633(g)) to deny benefit accorded Weissmann of his earlier filed foreign applications for failure to disclose the best mode (Paper No. 118). The motion was dismissed. See Paper No. 126.

3. Is Weissmann entitled to benefit of the April 3, 1980 and October 2, 1980 filing dates of his EPO and UK applications [I.E.1 and I.E.2]?

4. Did Goeddel et al. derive the invention from Weissmann?

5. Should Weissmann be allowed to amend his preliminary statement?

6. Did the APJ correctly deny the Weissmann motion to add counts 3-7[II.C.1]?

7. Did the APJ correctly deny the Goeddel et al. motion for judgment[I.C]?

8. Did the APJ correctly deny the Goeddel et al. motion to redefine[I.A.1 and I.A.2]?

9. Goeddel et al. belated motions attacking benefit based on lack of best mode.

In addition, the following motions are before us:

10. Weissmann's belated motion to file a supplemental §1.682 notice accompanied by the supplemental notice and record (Paper Nos. 232, 233 and 234, Opposition Paper No. 239, Reply Paper No. 241).

11. The Goeddel et al. and Weissmann motions to suppress and strike:

a. Goeddel et al. motion to strike or deny consideration of portions of Weissmann's motion under 1.682 or to return the Weissmann motion as an unauthorized paper (Paper No. 246). An opposition and reply were filed, (Paper Nos. 240 and 248).

b. Weissmann motion to suppress the Weissbach Dep. Ex. 30 (Paper No. 244) Opposition (Paper No. 251).

c. Goeddel et al. motions to suppress the Bullock memo (Weissmann Exhibit Bullock 1) and Gould memo (Goeddel Weissbach Dep. Exhibit 33) (Paper No. 236) Opposition (Paper No. 245) and Reply (Paper No. 249).

d. Goeddel et al. motion to strike the first Gilbert declaration (Paper No. 237) Opposition (Paper No. 242) Reply (Paper No. 247).

The voluminous Goeddel et al. record includes the declaration and deposition testimony of Herbert Weissbach, David Goeddel, Philip Familletti, Russell McCandliss, Linda Randall, Elizabeth Yelverton, Laurie May, Kate Murashige, Parkash Jhurani, Thomas Dull, Grace Ju, Sidney Pestka, Alan Sloma, Herbert Boyer, Peter Lomedico, Jacques Van Boom, Menachem Rubinstein, Kenneth Berkowitz, George Gould, Jordan Gutterman, Josef Leiter, Brian McCarthy, Lee Simon and Richard Stevenson together with numerous exhibits. The voluminous Weissmann record includes the declaration and deposition testimony of Edward Bailey, Werner Boll, Francis Bullock, Joan Gallagher, Walter Gilbert, Alfred Goldberg, James Haley, Alan Hall, Masayoshi Mishina, Phillip Sharp, Charles Weissmann and Hal Wolkoff together with numerous exhibits.[9]

[9] The Weissmann and Goeddel et al. records will be referred to as WR and GR. The parties briefs and exhibits will be referred to in a like manner, WB, GB, WX and GX, followed by the appropriate number.

Both parties filed briefs and appeared through counsel at final hearing. The question of no interference-in-fact has not been raised.

Issue (1)

In their brief, Goeddel et al. raised the dismissal of their motions I.D.1, I.D.2 and I.D.3 and the granting-in-part of Weissmann motions II.E.1 and II.F.

During a conference call on March 4, 1989 (pursuant to 37 CFR §1.610(d)) between the APJ and lead counsel for the parties, counsel for Goeddel et al. stated that, in addition to priority, the only issues to be raised by party Goeddel et al. at final hearing would be the denial of motions I.E.1. and 2., I.C. and I.A.1. and 2. The APJ acknowledged the issues Goeddel et al. wished to raise at final hearing in his interlocutory order of April 5, 1989 (Paper No. 109). The APJ further indicated in that order that "No other testimony may be taken and no other issues are entitled to be raised at final hearing."

The purpose of a conference call is to simplify issues and to consider such matters as may aid in the disposition of an interference. In accordance with 37 CFR §1.2, the APJ's order represents the written record of the conference call and each party is expected to adhere to any agreement or commitment made during the call. Notwithstanding the APJ's order, Goeddel et al.

assert that pursuant, to 37 CFR §§1.655(b) and (c), they are entitled to raise for consideration at final hearing the matters of I.D.1, I.D.2, I.D.3, II.E.1 and II.F because these matters were properly raised and the APJ improperly dismissed I.D.1, I.D.2, and I.D.3 and that such dismissal constitutes manifest error and/or an abuse of discretion or leads to manifest injustice.

Having stated that the only issues to be raised by them at final hearing would be the denial of the three above identified motions, Goeddel et al. waived their right to address matters presented either in their preliminary motions or oppositions to motions which were not raised by their opponent in its brief. To permit Goeddel et al. to do otherwise would vitiate the purpose of that procedure and be contrary to the spirit and purpose of the Rules, 37 CFR §1.601 et seq. If Goeddel et al. disagreed with any portion of the APJ's order, they could have, and in light of their subsequent actions should have, seasonably filed a request for modification within 14 days of the issuance of the order memorializing the conference call. No such request was filed.

An interlocutory order is presumed to have been correct. The Board may consider whether an interlocutory order is an abuse of discretion with the burden of showing such abuse

being on the party attacking the order. 37 CFR §1.655(a).[10] By raising these matters now, Goeddel et al. is attacking the APJ's interlocutory order of April 5, 1989. However, they have not shown, nor do they allege, any abuse of discretion on the part of the APJ with respect to that order. Nor do we find any.

For the foregoing reasons, we decline to consider the questions raised in motions I.D.1, I.D.2., I.D.3., II.E.1. and II.F.

Issue (2)

The Goeddel et al. case for priority

Goeddel et al. presented a case for priority premised upon simultaneous conception and reduction to practice of the subject matter of the count on May 16, 1980. See GB 92-128, GR and exhibits cited therein. Weissmann does not challenge the Goeddel et al. priority case. See WB 2-3, II. Issues presented. In fact, Weissmann alleges that judgment should be awarded to him solely because he is entitled to the April 3, 1980 filing date of his EPO application and "Goeddel's date--May 16, 1980--is far too late." See WB 25, III. first full paragraph. Hence, Weissmann

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[10] 37 CFR 1.655(a), has now been amended, 60 Fed. Reg. 14488-536 (March 17, 1995) and reads in part:

> All interlocutory orders shall be presumed to have been correct, and the burden of showing an abuse of discretion shall be on the party attacking the order.

has conceded the date of May 16, 1980 to Goeddel et al. and we will now address whether Weissmann is entitled to the filing date of April 3, 1980.

Issue (3)

Is Weissmann entitled to benefit of the April 3, 1980 and October 2, 1980 filing dates of his earlier filed EPO and UK Applications[11]

A.

Initially we point out that under the new rules, once an interference has been declared and a party seeks to change the status of the parties by motion, the moving party has the burden of persuasion to establish by a preponderance of the evidence that it is entitled to the relief sought. Kubota v. Shibuya, 999 F.2d 517, 519, n.2, 522, 27 USPQ2d 1418, 1420, n.2, 1422 (Fed. Cir. 1993). Hence, Goeddel et al. have the initial burden of establishing by a preponderance of the evidence that the Weissmann EPO application No. 80301100.6 filed April 3, 1980 [hereinafter April '80 EPO application] does not satisfy the

[11] In view of the fact that Weissmann has conceded to Goeddel et al. the date of May 16, 1980 for priority purposes, we do not find it necessary to address whether Weissmann is entitled to the benefit of the October 2, 1980 filing date of the UK application.

enablement requirement of 35 USC 112, first paragraph, with respect to the count.[12]

Weissmann argues that since the APJ has twice found that the April '80 EPO application enables the count, first upon the declaration of the interference and second when the Goeddel et al. motion was denied, that pursuant to 37 CFR §1.655(a), Goeddel et al. bear the burden to show that these two decisions by the APJ were an abuse of discretion.

We reject this argument. The starting point for declaring an interference is form PTO-850 filled out by the primary examiner. Thus, contrary to Weissmann's allegation, the primary examiner, not the APJ, initially determined that Weissmann was entitled to benefit of an earlier filed application(s) with respect to the count. See Manual of Patent Examining Procedure, §2309.02. When an interference is declared, rebuttable presumptions are created which the parties may challenge by filing appropriate motions. Orikasa v. Oonishi, 10 USPQ2d 1996, 2005 (Comm'r. 1989). Subsequent events in an interference, such as the filing of a preliminary motion, may

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[12] Goeddel et al. in their motion originally argued that the April '80 EPO application, despite the disclosure of the amino acid sequence of a single polypeptide of 166 amino acids corresponding to a mature human leukocyte interferon within count 1, namely IFN-α 1 (see original claims 25, 37, 43, 64, 70 and Fig. 8-10), did not satisfy the written description requirement of 35 USC 112, first paragraph, because the application failed to describe a method of making the compound. That argument has not been raised in their brief. Matters not raised in the brief are ordinarily considered abandoned. Photis v. Lunkenheimer, 225 USPQ 948, 950 (Bd. Pat. Int. 1984).

convince an APJ or a panel of the Board that an interference was not properly declared in the first instance. Here, the primary examiner accorded Weissmann benefit of April '80 EPO application on the PTO-850 and after the interference was declared, Goeddel et al. filed, inter alia, a preliminary motion to deny Weissmann benefit, 37 CFR §1.633(g). The APJ denied the Goeddel et al. motion. Since the parties have submitted additional testimony, evidence and arguments with respect to the issue of enablement making the record before us substantially different from that before the deciding APJ, we will not review the APJ's decision for an abuse of discretion, but rather we will decide whether the movant is entitled to the relief requested in view of the totality of evidence and arguments presented by the parties.

B.

We hold that the April '80 EPO application does not constitute a constructive reduction to practice of the invention of the count in that it fails to satisfy the enablement requirement of 35 USC 112, first paragraph. Accordingly, party Weissmann is not entitled to the benefit of April 3, 1980 filing date accorded it in the declaration notice.

In their brief, Goeddel et al. argue that the April '80 EPO application does not enable the count because the application itself fails to describe or suggest a method of making a compound of the count and one skilled in the art using the extant state of

art and the disclosure of the April '80 EPO application would not have been able to make a compound of the count without undue experimentation as of April 3, 1980. To support this position, Goeddel et al. rely principally upon the testimony of coinventor Goeddel (Dr. Goeddel), Professors Boyer and McCarthy. Goeddel et al. also rely upon the work performed by Drs. Mishina and Hall and Mr. Boll, coworkers of inventor Weissmann (Dr. Weissmann), after April 3, 1980 to demonstrate lack of enablement.

Contrarily, in his brief, Weissmann urges that the April '80 EPO application is enabled because a method of synthesis would have been obvious to one skilled in the art on April 3, 1980 from this application in combination with the Goeddel 1979 Nature article [hereinafter the Goeddel Nature article][13] and EPO application No. 1929 [hereinafter EPO 1929].[14] In support of this position, Weissmann relies upon the testimony of Dr. Weissmann, as well as that of Professors Gilbert, Sharp and Goldberg and the decision of Martin v. Johnson, 454 F.2d 746, 172 USPQ 391 (CCPA 1972) [An application is enabling even though it is "devoid of a disclosure of how to make the compound" if

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[13] Goeddel et al., Direct Expression in *Escherichia coli* of a DNA Sequence Coding for Human Growth Hormone, Nature, Vol. 281, pp. 544-548 (October 18, 1979).

[14] EPO No. 1929, a patent publication to Itakura et al., assigned to Genentech, Inc., published May 16, 1979. This publication is the equivalent of UK 2,007,676A, published May 23, 1979 and relied upon by Weissmann in his opposition paper.

"the method of synthesis would have been known to one of ordinary skill in the art."].

In order for a party to be accorded benefit of the filing date of an earlier application under 35 USC 119, the earlier application must satisfy the requirements of 35 USC 112, first paragraph, for an embodiment within the scope of the count, Bigham v. Godtfredsen, 857 F.2d 1415, 1417, 8 USPQ2d 1266, 1268 (Fed. Cir. 1988); Cross v. Iizuka, 753 F.2d 1040, 1043, 224 USPQ 739, 741 (Fed. Cir. 1985) and Kawai v. Metlesics, 480 F.2d 880, 891, 178 USPQ 158, 167 (CCPA 1973).

35 USC 112, first paragraph, provides in pertinent part:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same...

The test of enablement is whether any person skilled in the art could make and use the invention from the disclosure in the application coupled with information known in the prior art without undue experimentation. Hybritech Inc. v. Monoclonal Antibodies, Inc., 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986) cert. denied, 480 U.S. 947 (1987). Here enablement must be determined as of the April 3, 1980 filing date of the earlier filed EPO application. In re Wright, 999 F.2d 1557, 1563, 27

USPQ2d 1510, 1514 (Fed. Cir. 1993) [Events which occurred after filing date "are of no significance regarding what one skilled in the art believed as of that date."(footnote omitted)]. In re Glass, 492 F.2d 1228, 181 USPQ 31 (CCPA 1974). Each case must be considered on its own facts.

The count is directed to certain polypeptide products that are defined in terms of the process by which they are produced.[15] The products are polypeptides of about 165-166 amino acids comprising the amino acid sequence of a mature human leukocyte interferon without any corresponding presequence or portion thereof. As stated, the products are microbially produced, for example in bacteria such as E. coli, using recombinant DNA technology. The count, as drafted, actually embraces a genus of mature human leukocyte interferons, and includes the species disclosed in the respective parties' applications, to wit, Weissmann's species α-1 and α-2, the Goeddel et al. species, LeIFA, LeIFB, LeIFC, LeIFD, LeIFE, LeIFG, LeIFH, LeIFI and LeIFJ, allelic variations thereof, either glycosylated or unglycosylated, all in either mature or

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[15] A product-by-process claim normally is an after-the-fact definition, used after one has obtained a material by a particular process. Fiers v. Sugano, 984 F.2d 1164, 25 USPQ2d 1601 (Fed. Cir. 1993).

methionylmature form.[16] The count excludes fusion proteins and precursor forms of human leukocyte interferon by the phrase "unaccompanied by any corresponding presequence or portion thereof."

The April '80 EPO application

The April '80 EPO application indicates that interferons in general protect cells against a wide spectrum of viruses. Disclosed are two species of human interferon, fibroblast (F) produced in diploid fibroblast and leukocyte (LeIf) produced together with minor amounts of (F) in human leukocyte and lymphoblastoid cells, both have been purified and characterized. Of interest here, LeIf contains two components, the first is 21,000-22,000 in molecular weight and the second is 15,000 to 18,000 in molecular weight which appears to represent the non-glycosylated form of the former. Interferons are not detectable in normal or healthy cells but are produced as a result of the cells' exposure to an interferon inducer. As of April 3, 1980, human leukocyte interferon was produced either through human cells grown in tissue culture or through human leukocytes collected from blood donors. In addition to its anti-

[16] We have not been asked, nor do we find it necessary to address whether the claims of the parties embrace a naturally occurring product. This issue can and should be addressed ex parte after the termination of the interference.

viral action, the uses of human leukocyte interferon were wide ranging and these natural sources were not adequate to provide the needed quantities of human leukocyte interferon. Hence the discovery of alternative means for the production of human leukocyte interferon was desirable. See Background Art, pages 1-6.

The April '80 EPO application of Weissmann specifically describes a method for microbially producing a polypeptide which is trypsin sensitive, acid stable and has interferon-like activity.[17] Weissmann sequenced a DNA insert to a recombinant DNA molecule as set forth in Figures 8-10, determined the nucleotide bases representing the signal and mature sequences and deduced the amino acid sequence of the mature interferon therefrom. The polypeptide produced was sized and found to be "larger than authentic human leukocyte interferon, and the reason for that is almost certainly that it contains what is called a

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[17] The April '80 EPO application describes the preparation of the polypeptide product by preparing poly(A) RNA containing human leukocyte interferon mRNA from virus induced human leukocytes, preparing single-stranded complementary cDNA therefrom, converting the single stranded cDNA to double-stranded form, elongating the double-stranded cDNA with dCMP tails, annealing the tailed cDNA with a tailed vector, pBR322 cleaved at PstI site and tailed with dGMP tails, and transforming E. coli with the annealed product to make a library. Thereafter, the library was screened for a clone hybridizing to human leukocyte interferon mRNA by means of a hybridization translation assay. A clone designated Hif-2h was isolated and characterized. See Pages 14-51 of the application; WR 1097-1108, WR 263-266 and WR 820-822.

single [sic: signal] sequence." (Ju dec. Exh. 28, at B043522,[18] Weissbach Dep. Exh. 39, p. 116;[19] WR 1313)

The April '80 EPO application claims, inter alia, a polypeptide having a deduced amino acid sequence for a mature human leukocyte interferon, a compound within the scope of the count. See footnote 12, supra.

Weissmann acknowledges that his April '80 EPO application does not describe a method of making a compound of the count. See Paper No. 42, p. 20, GR 10669 and WR 1370.[20] The issue here is whether the description of an amino acid sequence of a mature human leukocyte interferon coupled with prior art methods of heterologous gene expression would have enabled one skilled in the art as of April 3, 1980 to microbially produce mature human leukocyte interferon without undue experimentation.

The term "undue experimentation" does not appear in 35 USC 112 but it is well settled that enablement requires that the

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[18] Transcript of Tape Recorded Press Conference in Boston, Mass. at the Park Plaza Hotel, January 16, 1980.

[19] Weissmann, "The Cloning of Interferon and Other Mistakes", Interferon, Vol. 3, pp. 101-134 (1981).

[20] Weissmann when questioned as to whether the EPO application contained a method of making a compound of the count stated:

> As a matter of principle, I had not introduced, to the best of my knowledge, protocols for things we had not done. And since we had at that time not really done anything which was specifically aimed at generating mature or met-mature interferon, I would not have put in such a protocol.

specification teach those in the art how to make and use the invention without undue experimentation. In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). Whether undue experimentation is needed is not a single, simple factual determination, but rather is a conclusion reached by weighing such factors as (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, and (8) the breadth of the claims. Id., 858 F.2d at 737, 8 USPQ2d at 1404 citing Ex parte Forman, 230 USPQ 546, 547 (Bd. Pat. App. & Int. 1986).

Weissmann states in the April '80 EPO application:

> [R]ecent advances in molecular biology have made it possible to introduce the DNA coding for specific nonbacterial eukaryotic proteins into bacterial cells. In general, with DNA other than that prepared via chemical synthesis, the construction of such recombinant DNA molecules comprises the steps of producing a single-stranded DNA copy (cDNA) of a purified messenger RNA (mRNA) template for the desired protein; converting the cDNA to double-stranded DNA; linking the DNA to an appropriate site in an appropriate cloning vehicle to form a recombinant DNA molecule and transforming an appropriate host with that recombinant DNA molecule. Such transformation may permit the host to produce the desired protein. (page 7, paragraph 1) (emphasis added)

Two publications which reflect these "recent advances" in this nascent technology are EPO 1929 and the Goeddel Nature article.

EPO 1929 is directed to the microbial production of human polypeptides, and in particular, somatostatin and insulin. Somatostatin, an inhibitor of the secretion of growth hormone, insulin and glucagon, is a polypeptide of 14 amino acids (page 6, lines 8-13). The process included chemically synthesizing a nucleotide sequence coding for the 14 amino acid polypeptide with an ATG start codon and fusing that sequence to the beta-galactosidase gene, placing the construct in an expression vector and transforming E. coli. The product was expressed as a fusion protein comprising a large portion of beta galactosidase and the met-somatostatin protein. This product was cleaved with cyanogen bromide at the methionine residue to release the active somatostatin protein product.

With respect to insulin, a human polypeptide of 51 amino acids, the process is similar to that of somatostatin and involved chemically synthesizing (1) a nucleotide sequence coding for the 21 amino acid human insulin A chain and (2) a nucleotide sequence coding for the 30 amino acid human insulin B chain. A ATG start codon was positioned before the first codon of the nucleotide sequences coding for the respective chains. The two separate synthetic genes were fused to a beta galactosidase gene in a vector under the control of lac operon elements. E. coli

were transformed with this construct. The products were expressed as individual fused proteins having a large number of amino acids of beta galactosidase fused to the respective A and B chains. Each of the fused polypeptides produced were partially purified, cleaved at their methionine residue with cyanogen bromide to release separate A and B chains which were then purified and recombined to form the 51 amino acid human insulin.

The Goeddel Nature article describes a process of microbially producing human growth hormone HGH, a protein of 191 amino acids in length, which is synthesized in the anterior lobe of the pituitary. Dr. Goeddel testified in regard to his HGH work that he turned to cDNA to obtain HGH because he did not find it feasible to chemically synthesize a protein of such large size. Dr. Goeddel also indicated that he did not want to produce a fusion protein since (1) such a fusion protein would not be pharmaceutically useful and (2) the HGH could not be released from the fusion protein using the prior art cyanogen bromide technique due to the number of internal methionine residues apart from the methionine resulting from the ATG start codon. (GR 403-404, 408-409).[21]

[21] Somatostatin and insulin do not contain methionine residues except for the inserted start codon.

The Goeddel Nature article describes in great detail the hybrid cloning technique and expression of mature HGH as met-HGH. The article indicates that after first cloning the cDNA from human pituitary mRNA, sequencing the insert, and determining the restriction endonuclease pattern, a double stranded HGH cDNA was treated with HaeIII giving a DNA fragment of 551 base pairs which included coding sequences for amino acids 23-191+ of HGH. To complete the needed nucleotide sequence, a DNA adaptor fragment containing an ATG initiation codon and coding sequences for residues 1-23 of HGH was chemically synthesized. The two fragments were separately cloned and then combined to form a synthetic-natural 'hybrid' gene which was then modified with appropriate ends in order to ligate it to a prepared plasmid. The prepared plasmid was modified to contain tandem lac UV-5 promoters and appropriate ends to accept the 'hybrid'. In addition, the promoter sequence of the tc^R gene and distal EcoRI were removed from the plasmid. The synthetic-natural 'hybrid' gene was ligated into the prepared plasmid and positioned with respect to the promoter and ATG codon. This construct was then transformed into various E. coli extracts. See Table 1. The plan to express HGH included positioning two base pairs between the EcoRI sticky end and the ATG initiation codon such that the lac AGGA ribosome binding site was eleven (11) base pairs from

the ATG translational start for HGH. Other prepared plasmids positioned the ribosome site seven (7) base pairs from the ATG start codon. The Goeddel Nature article, at page 548, states that

> This is the first time that a human polypeptide has been directly expressed in E.coli in a non-precursor form.. The hybrid DNA cloning techniques described as a route to the cloning and expression of HGH coding sequences in E.coli are generally applicable to other polypeptides which are synthesized initially as inactive precursors and later processed, or for which full length cDNA transcripts are unavailable. (emphasis added)

We hold that Goeddel et al. have sustained their burden of proof to establish by a preponderance of the evidence that the April '80 EPO application of Weissmann is not enabling with respect to making a compound of the count. As admitted by Weissmann, his April '80 EPO application does not disclose any method for making a compound of the count. Nor does it direct one skilled in the art to other prior art disclosures for the needed information. As of April 3, 1980, the prior art did not disclose even in general terms how to microbially produce mature or met-mature human leukocyte interferon.

As is clear from the April '80 EPO application, as of April 1980, the ability of workers to express human proteins in bacteria was a very recent advance in molecular biology, i.e., the field was in its infancy. At that time, researchers were not

directly expressing heterologous genes in bacteria in a routine manner. As explained by Boyer, as of April 3, 1980, researchers in the art of molecular biology were seriously concerned that attempts to produce specific mature mammalian proteins in bacteria would be fraught with problems since bacteria simply do not ordinarily produce mammalian or other eukaryotic proteins and there are major differences that render efforts to express specific mammalian proteins in bacteria unpredictable. (GR 2069-2077, ¶¶ 39-50). Boyer lists a number of concerns (GR 2070-2071) in expressing mammalian proteins in E. coli, including (1) instability of the mammalian gene and the recombinant expression vector containing it in the bacteria cell, (2) inability of the bacterial cell to transcribe the mammalian gene, (3) instability of mRNA encoding the mammalian protein in the bacterial cell, (4) inability of bacteria to translate the mammalian mRNA into protein, (5) instability of the mammalian protein in the bacteria environment, (6) inability of the bacterial cell to fold the mammalian protein into its proper three-dimensional conformation; and (7) instability of the biological activity of the bacterially-produced mammalian protein. (See also GB 83-86). Boyer testified that these concerns "would lead one to say that you couldn't predict with any degree of certainty that any construct is going to make a particular protein."(GR 2184). Unpredictability of an art area alone can lead to a conclusion of

nonenablement. In re Marzocchi, 439 F.2d 220, 169 USPQ 367 (CCPA 1971); In re Fisher, 427 F.2d 833, 166 USPQ 18 (CCPA 1970).

In rebuttal, Weissmann urges that the Goeddel Nature article and EPO 1929, especially as interpreted by Genentech in its paper filed December 20, 1990 in an opposition proceeding in the European Patent Office, provide the necessary information to make a compound of the count. We disagree. We find it significant that these two publications were in fact disclosed in the April '80 EPO application, yet the April '80 EPO application, while describing the deduced amino acid sequence of the mature human leukocyte interferon, does not state that the techniques described in these two publications would be useful for the production of human leukocyte interferon. To the contrary, Weissmann stated in the April '80 EPO application that "[N]one of the foregoing [publications] however is directed, as is this invention, toward the synthesis of HIF with use of recombinant DNA technology."

The contemporaneous statements made in the April '80 EPO application by Weissmann are relevant evidence establishing that Weissmann did not think, as of April 3, 1980, that these two publications were at all significant or relevant to the production of human leukocyte interferon in contrast to his position in this interference. In our view those contemporaneous statements are entitled to significantly more weight than

Weissman's belated reliance upon these two documents after this interference was declared. Thus on its face, the April '80 EPO application does not provide any guidance with respect to the direction one should proceed to produce a compound of the count. In fact, by distancing itself from the two publications Weissmann now relies upon, the April '80 EPO application can reasonably be read as directly teaching away from the procedure Weissmann now argues would have been applicable.

Weissmann points to the prophetic statement made at page 548 of the Goeddel Nature article and alleges that it provides a reasonable basis to conclude that that method is applicable to the microbial production of other polypeptides initially made in precursor form, such as interferon. We do not read "generally applicable" as the phrase is used in this prophetic statement to mean that at that embryonic stage of this art that novel hybrid cloning technique would necessarily work with any other specific protein.

In fact, Dr. Goeddel testified that it was not possible to use the Goeddel Nature article method to tailor the DNA fragment for fibroblast (beta) interferon. (GR 315-316 ¶35). Rather, Dr. Goeddel needed to develop another method for the

production of DNA construct of fibroblast (beta) interferon.[22] Weissmann himself, when questioned about the Goeddel Nature article and the usefulness of the Goeddel techniques for proteins other than HGH, said "...the methods described in this paper are generally applicable to other polypeptides which are synthesized as inactive precursors and later process [sic, processed]" (emphasis added) (WR 1122). Boyer, referring also to the Goeddel Nature article, indicated that as far as the HGH method was concerned, there were two things to consider, the first was the "general applicability" in terms of making a construct and the second was whether or not one can predict whether one could successfully produce a protein from the construct (GR 2155). Boyer further stated in respect to the HGH method that "one can go ahead and try it, there is no guarantee it will work." (GR 2159) and that "the uncertainty was whether or not one could have the construct make the appropriate protein." (GR 2159). Weissbach testified that the HGH information "was but a starting place to try to express a protein, one could not be sure whether it would work." When questioned why, Weissbach explained that it was a complicated process which depended in large part on the sequence of the protein. Each protein is different and one could

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[22] See Goeddel et al., "Synthesis of Human Fibroblast Interferon by E. coli," Nucleic Acids Research, Vol. 8, No. 18, pp. 4057-74 (September, 1980). Goeddel First Declaration Ex. C.

not be sure that one could use this procedure to construct a proper high expression clone. In addition, there are many other steps involved between the time a clone is obtained and actual synthesis of the protein by an organism. (See GR 191-192). Dr. Goeddel himself stated that having the sequence and HGH method is not enough, i.e., that only with that information is it possible to design a method to try to directly express. The information shown in the Goeddel Nature article does not tell you how to "do interferon." (See GR 454-455).

Weissmann also relies upon the opinion testimony of Professors Gilbert and Sharp (Gilbert and Sharp) (WR 268-272 and 822-829) to show that, as of April 3, 1980, a person skilled in the art could have been able to produce a mature human leukocyte compound of the count having knowledge of the April '80 EPO application containing the method of producing a polypeptide having interferon activity, the nucleotide sequence of Dr. Weissmann's cDNA and the identity of the first codon of mature human leukocyte interferon of that DNA sequence, combined with the technique for expressing a mature form of a polypeptide disclosed in the Goeddel Nature article. Gilbert and Sharp set forth the following steps:

(a) make and isolate a cDNA encoding pre-human leukocyte interferon;

(b) determine the DNA sequence of that cDNA and locate the N-terminal of the mature human leukocyte interferon coded for it;

(c) restrict that cDNA with a restriction endonuclease that cuts in the N-terminal portion of the coding region for mature human leukocyte interferon;

(d) synthesize a DNA linker having, in the 5' to 3' direction:

(i) an ATG codon, and
(ii) the excised portion of the mature human leukocyte interferon cDNA;

(e) ligate the synthetic linker and the restricted cDNA into a microbial expression vector at an appropriate position for expression; and

(f) transform an appropriate microbial host with the expression vector containing the synthetic DNA-cDNA hybrid and culturing that host.

With respect to these various steps, Gilbert and Sharp indicate that steps (a) and (b) are found in the April '80 EPO application. As to step (c), they indicate that restriction endonucleases, their sites and methods for restricting DNA with them were well known in February 1980. They specifically refer to the use of DpnII (MboI) (or Sau3A), DdeI or AvaII, all of which have recognition sequences within the N-terminal portion of the mature coding sequence of human leukocyte interferon as of April 3, 1980. For step (d), linkers could be ordered and purchased at this time. As to steps (e) and (f), Gilbert and

Sharp assert that ligation, transformation and culturing were "standard and highly predictable techniques in the field of genetic engineering" in February 1980. Specific examples of these techniques were described by Dr. Weissmann at the Martinique meeting and the MIT seminar, in the Nagata Nature paper ...and in the April 1980 EPO application."

While these individual steps and techniques may have been known at that time, in view of the embryonic nature of this field and the lack of guidance in the specification, it is difficult to find a reasonable basis to conclude that one would have obtained expression of the protein by these techniques without further guidance as to the direction that experimentation should take. While we recognize that a production specification is not required to satisfy the enablement requirement of 35 USC 112, first paragraph, the purpose of this section of the statute is to make an invention fully available to the public without any requirement of undue experimentation. Cf. Martin v. Johnson, supra, wherein a organic chemist provided a detailed explanation of how a person skilled in the art would have prepared 3-(p-bromo-phenyl)-1-methyl-1-methoxyurea by using p-bromophenyl isocyanate as a reactant instead of p-chlorophenyl isocyanate in the reaction of p-chlorophenyl isocyanate with O,N-dimethyl hydroxylamine in benzene. Here, experimentation is needed to determine at a minimum appropriate ligation sites, appropriate

cloning molecules to form the recombinant DNA molecule, appropriate hosts, etc. As noted by Weissmann himself in the April '80 EPO application, even if one fortuitously selects appropriate materials, expression is not guaranteed, only that with appropriate selections "transformation may permit the host to express." See the April '80 EPO application at page 7, lines 12-13. Boyer stated that "every protein had its own series of problems relating to expression."(GR 2165). Hence, we conclude that the Gilbert and Sharp testimony does not establish that one skilled in the art would have been able to practice the count without undue experimentation.

Weissmann would also have us look to the April '80 EPO application and its 55 pages of examples disclosing "how to produce biologically active interferon microbially." This argument is not relevant in that the product which is actually produced in the application is not a compound within the scope of the count.

Weissmann directs us to the Goeddel Nature article to show detailed, actual examples of making a mature form of HGH.[23]

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[23] It would appear that one example in Table 1 would appear to support the Goeddel et al. position of unpredictability in the art at this time. In that Table, there is one extract which does not result in any HGH activity in the radioimmunoassay. No explanation is provided for the lack of activity. However, Dr. Goeddel when questioned with respect to the lack of activity in a radioimmunoassay of somatostatin, postured that the most likely reasons for the inability to detect protein are no synthesis of the protein or very rapid degradation.(GR 390).

However, Weissmann has not addressed any specific example with any reasonable degree of specificity nor has he provided testimony which specifically analyzed the expression method therein or explained how that method could be directly applied to interferon. Cf. Martin v. Johnson, supra.

Weissmann further directs our attention to the Goeddel et al. patent application on HGH filed in 1979 and issued as U.S. Pat. No. 4,343,832 on August 3, 1982 (WR 9730-9742) for a teaching that the method of the Goeddel Nature article was particularly useful for the production of mature interferon. This patent cannot be used to show the state of the art as of the April 3, 1980 filing date since it issued thereafter and thus it is inappropriate for us to consider it. In re Glass, 492 F.2d at 1231, 181 USPQ at 34 [A patent may be available as prior art under 35 USC 102(e), however it does not show what is known generally to any person skilled in the art as of its filing date.]

Weissmann also directs us to the Swanson and Leibowitz statements, found in the Gould memo (WX Sharp 14) and the Bullock memo (WX Bullock 1), respectively, alleging that these statements also establish that the method in the Goeddel Nature article is applicable to interferon. Weissmann relies upon the following statement attributed to Swanson, president of Genentech, that "they had an exciting breakthrough regarding human growth hormone

production by recombinant-DNA techniques which they believe would be applicable to the interferon project." The Swanson statement, in our view, does not reflect what one skilled in the art knew at the time of filing of the April '80 EPO application. Moreover, these statements indicate no more than the prophetic statement contained in the Goeddel Nature article that technique is generally applicable to other polypeptides. The Bullock memo is not before us.[24]

Weissmann has also proffered the Genentech paper (WR 111747-11781) dated December 20, 1990 and filed in an EPO opposition proceeding involving EPO 1929, alleging that in that paper, Genentech, the Goeddel et al. coassignee, interprets EPO 1929 to describe microbial production of human proteins "as such" unfused to any other protein. WB 15-16, 32. We give little weight to this evidence since the statements made by Genentech in 1990 are not revelant to what one skilled in the art believed as of April 3, 1980. Moreover, we do not find any basis upon which to conclude that the EPO 1929 application teaches one of ordinary skill in the art how to express heterologous genes in bacteria without a presequence since the disclosure and examples therein

[24] The Bullock memo, dated May 19, 1980, containing the statements of Leibowitz and Ryan was subject to a motion to suppress which has been granted, see infra. The evidence is not properly before us, and even if it were, it would not be relevant since it is not reflective of what one of ordinary skill in the art knew as of April 3, 1980.

are all directed to the production of fusion proteins which are later cleaved to release the mature protein.

We are not persuaded by the allegations of Weissmann's witnesses that all of the concerns iterated by Boyer relating to stability and degradation dissipate simply because the April '80 EPO application describes microbial production of a polypeptide having interferon activity. That polypeptide product is not within the scope of the count and the Weissmann witnesses have not presented a proper factual basis, only opinion, to conclude that one skilled in the art would have successfully extrapolated the April '80 EPO application technique for producing a polypeptide having interferon activity to producing the mature human leukocyte interferon of the count. Moreover, stability is a nonissue unless protein expression occurs. In addition, the Goldberg opinion of the Taniguchi et al. article[25] appears, in our view, to be inconsistent with his position that one of ordinary skill realized that smaller proteins are less stable

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[25] Taniguchi et al., "Expression of the Human Fibroblast Interferon Gene in Escherichia coli," Proc. Natl. Acad. Sci, Vol. 77, No. 9, pages 5230-33 (September 1980) (Goeddel Second Declaration, Ex. D).
This article reports that upon pulse-chasing human fibroblast pre-interferon containing 187 amino acids is completely degraded at 50 minutes and the mature fibroblast containing 166 amino acids is 50% degraded after 50 minutes. Both parties rely upon this article alleging that it supports their respective positions. Goeddel et al. argues that this article shows that stability of one form of protein is not predictive of stability of another form of that protein (GR 2077-78, GB 86 n.58). Goldberg opines that this article shows that the mature protein is more stable than its corresponding preprotein and thus one would expect the mature human leukocyte interferon to be more stable than the preprotein (WR 430-431).

than large mammalian proteins. EPO 1929 indicates that size is relevant to survival. Taniguchi et al. shows that both the larger preprotein and smaller mature fibroblast protein degrade. Both EPO 1929 and the Goeddel Nature article evince degradation concerns with their respective proteins. Further, Weissbach testified that stability was a definite concern in this new area of research because people were not expressing eukaroytic proteins in bacteria (GR 261-262) and that one could not predict degradation (GR 263). This is further evidence of the uncertainty as to whether microbial expression of interferon as of April 3, 1980 would have been successful.[26]

In holding that Goeddel et al. sustained their burden and that the Weissmann evidence is not an adequate rebuttal, it should be noted that we give little weight to the work done by Drs. Mishina and Boll and Mr. Hall, coworkers of Dr. Weissmann,

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[26] The fact that stability and degradation were concerns as of April 3, 1980 is also supported by the April '80 EPO application itself, page 23, which indicates that the selection of vectors and hosts is determined by "susceptibility of the desired protein to proteolytic degradation by host cell enzymes". Stability also appeared to remain a concern at the time testimony was taken. Dr. Weissmann, himself testified when asked what effect the differing stabilities of preinterferon fibroblast and mature fibroblast interferon have upon the predictability of the stability of mature alpha interferon and met-mature alpha interferon in E. coli, that he could not conclude anything from the Taniguchi publication because "certainly at that time, and even today very little is known about what makes a protein more or less stable in E. coli, and the differences between fibroblast interferon and leukocyte interferon were too substantial to allow any reasonable deductions to be made from one to the other." (WR 1137). Dr. Weissmann's testimony would appear to be in conflict with Goldberg's view that Taniguchi et al. supports his view.

after the filing date of the April 3, 1980. Goeddel et al. urge that this evidence shows that a number of attempts were unsuccessful, that Weissmann was not able to produce a mature interferon of the count until October 21, 1980 and that even this successful attempt did not use the exact procedure of the Goeddel Nature article but, rather, Dr. Weissmann used a variant thereof.[27] Goeddel et al. argue that this evidence establishes nonenablement as of April 3, 1980, that is, one skilled in the art would not have been able to use the Goeddel et al. Nature article procedures to produce mature human leukocyte interferon as it was used to produce HGH without undue experimentation. Not surprisingly, Weissmann relies upon this same work as evidence that he successfully microbially produced a compound within the scope of the count by October 21, 1980. We have given this evidence little weight in reaching this decision since it is of little, if any, relevance to the question of enablement. Neither party has established that these developments which occurred

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[27] Weissmann deviated from the procedure disclosed in the Goeddel Nature article in that he synthetically attached the first codon, TGT, via the HindIII (Wu) linker, to the mature sequence which had been partially digested with a restriction endonuclease (Sau3A), to rebuild the mature sequence. He did not ligate ATG to the mature sequence as in the Goeddel Nature article, but rather used a linker oligonucleotide, provided by Collaborative Linker, to provide an ATG start codon to the lac promoter. The two constructions were ligated to provide a lac promoter flanked on its downstream side by an EcoRI site, a small piece of plasmid and a HindIII site flanked by the mature coding sequence, this construct was then cleaved with EcoRI and HindIII and digested to remove the overhanging ends, fused to position the promoter-ATG to the first codon of the mature sequence. (WR 1204-1214)

after the effective filing date of the April '80 EPO application are of significance in determining what one skilled in the art believed as of that date. Enablement is determined as of the April 3, 1980 filing date of the patent application. In re Wright, supra; In re Glass, supra.

For the above reasons, we find that Goeddel et al. have sustained their burden and Weissmann's rebuttal is ineffective.

Issue (4)

Did Goeddel et al. derive the invention of the count from Weissmann

Since Weissmann is not entitled to benefit of the April 3, 1980 filing date, the only way that Weissmann may prevail is to prove derivation. We hold that Goeddel et al. did not derive the invention of the count from Weissmann by April 15, 1980 as alleged by Weissmann.

The party charging derivation has the burden of showing prior complete conception of the subject matter of the count and sufficient communication of the subject matter to the party charged to enable one of ordinary skill in the art to construct and successfully operate the invention. Mead v. McKirnan, 585 F.2d 504, 507, 199 USPQ 513, 515 (CCPA 1978). Conception is "the formation in the mind of the inventor, of a definite and permanent idea of the complete and operative invention, as it is

hereafter to be applied in practice." Hybritech, supra, 802 F.2d at 1376, 231 USPQ at 87. Conception is complete only when the idea is so clearly defined in the inventor's mind that only ordinary skill would be necessary to reduce the invention to practice, without extensive research or experimentation. Sewall v. Walters, 21 F.3d 411, 415, 30 USPQ2d 1356, 1359 (Fed. Cir. 1994). Since the applications are copending, the burden is on Weissmann to prove his case by a preponderance of the evidence. Davis v. Reddy, 620 F.2d 885, 888, 205 USPQ 1065, 1068 (CCPA 1980).

For derivation, Weissmann (WB 46-47) alleges that (1) Goeddel et al. had a copy of the Weissmann Nagata Nature preprint (WX Haley, X5048-5080) in their files (2) Goeddel learned of the DNA and amino acid sequence of Weissmann's mature interferon by telephone from Roche's Weissbach and (3) Goeddel et al. had a copy of the DNA and amino acid sequence of Weissmann's leukocyte interferon as evidenced by their appearance in the Yelverton notebook.

While Weissmann presented no evidence to establish that Goeddel et al. was in possession of the Nagata preprint as of April 15, 1980, that is of no moment, since the Nagata preprint itself was published March 27, 1980 and therefore available as

prior art. This publication[28] describes Weissmann's microbial production of a polypeptide having interferon activity but does not disclose a DNA or amino acid sequence for the polypeptide. In any event, the polypeptide described in Nagata is not a compound within the scope of the count.

We agree with Weissmann that at least by April 15, 1980, Goeddel et al. were in possession of the DNA sequence and the corresponding deduced amino acid sequence which codes for Weissmann's mature interferon as evidenced by the presence of a pasted copy of the DNA and deduced amino acid sequence of mature human leukocyte interferon identified as "Weissmann huleukIF sequence" in the notebook of Yelverton, a technician in the laboratory of David Goeddel. See Yelverton testimony (GR 940-942) and undated notebook page 45980 (GX Yelverton Cross Exhibit 34). Note that page 45983 is dated 4-15-80. Thus, the issue is whether a description of a compound by itself, that is, the amino acid sequence of mature human leukocyte interferon, is sufficient to establish conception.

Weissmann has provided no evidence to show that a method of making a compound within the scope of the art was well known. Rather, he relies upon the combination of (1) the method

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[28] Nagata et al., "Synthesis in E. coli of a Polypeptide with Human Leukocyte Interferon Activity," Nature, Vol. 284, pp 316-320 (March 27, 1980). WX Gilbert 6.

of making a polypeptide having interferon activity, a compound outside the scope of the count found in Nagata publication, (2) the possession by Goeddel et al. of Weissmann's DNA sequence and corresponding deduced amino acid sequence, (3) the Goeddel et al. Nature article or EPO 1929 publication to establish that one skilled in the art would have been enabled to microbially produce a compound of the count.

Conception of a compound requires both the idea of the invention's structure and possession of an operative method of making it. Oka v. Youssefyeh, 849 F.2d 581, 583, 7 USPQ2d 1169, 1171 (Fed. Cir. 1988). When, as is often the case, a method of making a compound with conventional techniques is a matter of routine knowledge among those skilled in the art, a compound has been deemed to have been conceived when it was described, and the question of whether the conceiver was in possession of a method of making it is simply not raised. Id. In this instance, we do not find that possession of the amino acid sequence is sufficient to establish conception of the count for the reasons set forth above in regard to the enablement issue. Simply put, this record does not establish that one skilled in this art would have been able to make a compound of the count as of April 3, 1980 without undue experimentation. Thus, whether in the context of the enablement of the April '80 EPO application or the purported derivation of the invention from Weissmann by Goeddel et al.,

knowledge of the nucleotide/amino acid sequences by themselves or in the context of the Goeddel Nature article or EPO 1929 would not have enabled one skilled in the art to make mature human leukocyte interferon of the count without undue experimentation.

Having found no conception, we find no derivation.

Issue (5)

The Weissmann motion to amend his preliminary statement.

The motion is dismissed as moot.

In his original preliminary statement (Paper No. 29), Weissmann stated, in paragraph 14, that the earliest date that he had communicated his invention to opponents was at an April 15, 1980 seminar in Paris. In paragraph 14 as amended, in the motion to amend (Paper No. 171), Weissmann now alleges four dates earlier than April 15, 1980 where he allegedly communicated his invention to his opponents.

Even assuming arguendo that Weissmann (1) satisfied the requirements of 37 CFR 1.628(a) to correct the preliminary statement and (2) relied upon and proved the earlier alleged dates for communication, he would be in no better position with respect to the alleged earlier dates since he has not established conception.

Issue (6)

The APJ's denial of the Weissmann motion to add counts 3-7 to this proceeding. [IIC1]

Weissmann brought this motion (Paper No. 26), alleging that the restriction requirements made by the primary examiners in the involved Goeddel et al. and Weissmann applications during ex parte prosecution establish that counts 3-7 are separately patentable and thus they should be added to this proceeding. The APJ denied the motion stating that Weissmann had not sustained his burden of proof to establish separate patentability of the counts.

In their brief, Weissmann reiterate the same position set forth in the motion paper. We have reviewed the APJ's decision, however we find no abuse of discretion. Restriction practice is discretionary on the part of the PTO. Pointing to the prosecution history to show what the primary examiner did during ex parte prosecution does not satisfy the burden of the movant to show separate patentability within the meaning of 37 CFR §1.601(n). As noted by the rules, the standard of separate patentability is that of novelty and obviousness not that of restriction. Weissmann has not addressed this issue. The decision of the APJ is affirmed.

Issue (7)

The APJ's denial of the Goeddel et al. motion for judgment on the ground that Weissmann claims 37-39 and 41 and 42 are unpatentable over the Goeddel Nature Paper and Streuli Science Paper

The motion is dismissed as moot in view of our holding in Issues (2), (3) and (4), supra.

We make one additional observation and comment. In the brief, Goeddel et al. now allege that Weissmann claims 37-39, 41 and 42 are unpatentable over the Goeddel Nature article alone, in view of a public lecture by Heynecker at a September 8-9, 1980 NIH Interferon workshop [the Heynecker speech] (WR 1227; WX 67 at B35063) in combination with Streuli Science Paper (WX Weissbach 37). Goeddel et al. are now alleging for the first time in their brief that these claims are unpatentable over the Heynecker speech. The reliance on the Heynecker speech is new, raised for the first time in the Goeddel et al. brief. Hence, reliance on the Heynecker speech is improper, not entitled to any consideration by the Board and is therefore dismissed.

Issues (8) and (9)

The APJ's denial of the Goeddel et al. motion to designate Weissmann et al claims 37-39 and 41 and 42 and Goeddel claims 4 and 8 as not corresponding to count 1. [IA1 and IA2] and the Goeddel belated motions for judgment on the grounds of lack of best mode.

Consideration of these issues is deemed moot in view of the disposition of the issues of priority, enablement and derivation.

Issues (10) and (11)(a)

Weissmann's motion to file a belated supplemental §1.682 notice and to supplement the Weissmann record and the Goeddel et al. motion to strike or deny consideration to portions of Weissmann motion or to return the motion as an unauthorized paper.

The Weissmann motion is granted and the Goeddel et al. motion is denied.

On February 22, 1991, Weissmann moved to file a supplemental notice listing a paper dated December 20, 1990 and filed by Genentech, co-assignee of the involved Goeddel et al. application, in the European Patent Office during an opposition proceeding relating to EPO 1929 and to include the paper in his record.

Goeddel et al. object to the filing of the supplemental notice and to the entry of the paper for two reasons (1) the notice is untimely since it was filed after the close of the Weissmann testimony period and (2) a certified copy of the official record was not filed with the motion.

As to (2), Weissmann has now filed the certified copy (Paper No. 252) and the Goeddel et al. objection is moot. As to (1), Goeddel et al. point out that the motion was filed the day

before Goeddel et al. was to file its main brief. Goeddel et al. urge that it is manifestly unfair to be faced with this new document at this time since they were denied the right to present rebuttal evidence and deprived of an opportunity to consider the full scope of the evidence when filing their brief.

We agree with Weissmann that the document was submitted as soon as possible after it was received. The allegations of Goeddel et al. as to their purported denial to present rebuttal or time to consider the evidence when filing the brief are not persuasive arguments since Goeddel et al. could have requested an extension of time to consider the evidence before filing their brief or requested an opportunity to present rebuttal evidence by requesting that their testimony period be reopened for that purpose. No such request was made.

Therefore, the evidence will be entered for the purpose of rendering a complete decision.

The Goeddel et al. motion to strike or deny consideration to portions of Weissmann motion or to return the motion as an unauthorized paper is denied. The Weissmann motion is not an unauthorized paper.

Issues (11)(b), (c) and (d)

(11)(c) and (d) Goeddel 's Motion to Suppress

The Goeddel et al. motion moved to suppress (1) the first declaration of Walter Gilbert executed March 18, 1988, (2) Weissmann Exhibit Bullock 1, a memorandum dated May 19, 1980 authored by Drs. Leibowitz and Ryan as inadmissible hearsay, and (3) a memorandum by George Gould identified, inter alia, as Weissmann Exhibit (Haley) 33, Weissmann Exhibit (Sharp) 14 and Goeddel (Weissbach) Deposition Exhibit 33 as inadmissible non-authenticated hearsay.

As to (1), the motion is dismissed as moot since Weissmann acknowledges that the inclusion of the first Gilbert declaration was inadvertent and that Weissmann would not and have not relied on the declaration. Nor have we.

As to (2), the motion is granted for essentially the reasons set forth in the Goeddel et al. motion and associated reply. Weissmann does not challenge the Goeddel et al. charge of hearsay. Rather he urges that the memo is admissible under FRE 803(6) as a record of regularly conducted activity and that Bullock became the custodian of the memo when Liebowitz left the employ of Schering. However, the record clearly indicates that the memo before us is not the memo from the Leibowitz files to which Bullock was custodian but rather a memo that Bullock

supposedly received from Daniels, annotated and placed in his own files. To the extent that Weissmann relies upon the memo for the opinions of Leibowitz and Ryan, who did not testify, the exhibit is hearsay. Contrary to the Weissmann allegations, it does not qualify as a record of regularly conducted activity (FRE 803(6)) for the reasons set forth in the Goeddel et al. reply (Paper No. 249).

As to (3), the motion is denied. The exhibit is a memo dated July, 1979 addressed to Dr. J.J. Burns, by George Gould, then Assistant Patent Counsel for Roche, co-assignee of the involved Goeddel et al. application in which Gould iterates statements made to him in telephone conversations by Bob Swanson, the president of co-assignee Genentech. Goeddel et al. themselves introduced this memo into the record during their testimony. (GR 189 and 195). Goeddel et al. now object to the admission of the exhibit on the ground that by choice Gould was not cross-examined with respect to the memo and that the memo is inadmissible, non-authenticated hearsay. Weissmann allege that the Gould memo is admissible as admissions of a party opponent. FRE801(d)(2(A).

It is inappropriate for Goeddel et al., simply because Weissmann relies upon it, to move to suppress evidence which they themselves introduced into the record. Cf. In re Hedges, 783 F.2d 1038, 228 USPQ 685 (Fed. Cir. 1986) [An applicant supplying

references cannot object to the PTO's citation of other portions of the same reference].

(11)(b). Weissmann motion to suppress

Weissmann moved to suppress Goeddel Weissbach Deposition Exhibit 30, a document entitled "FDA Seminar on the State of the Art in Recombinant DNA Research", dated June 4, 1980 as hearsay.

Since we have not relied on this document in making our determinations, we find it unnecessary to address the Weissmann motion to suppress.

Decision

In view of the foregoing, priority is awarded to Goeddel et al. On this record, Judgment is awarded against Weissmann, and Weissmann is not entitled to a patent containing claims 37-39, 41, 42, 77-79, 85 and 87 corresponding to the

count. Judgment is awarded to David V. Goeddel and Sidney Pestka, and they are entitled to a patent containing claims 1-4, 8 and 52-69 corresponding to the count.

RONALD H. SMITH)
Administrative Patent Judge)
)
)
MARY F. DOWNEY)
Administrative Patent Judge) BOARD OF PATENT
APPEALS
AND
INTERFERENCES
)
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Paper No. 38

UNITED STATES PATENT AND TRADEMARK OFFICE

1803-097

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

Ex parte DAVID V. GOEDDEL
and ROBERTO CREA

Appeal No. 94-2099
Application 07/365,284[1]

HEARD: August 9, 1994

Before WINTERS, WILLIAM SMITH and GRON, Administrative Patent Judges.

WINTERS, Administrative Patent Judge.

DECISION ON APPEAL

This appeal is from the examiner's decision refusing to allow claims 25, 28-30, 32, and 33, which are all of the claims remaining in the application.

[1] Application for patent filed June 12, 1989. According to applicant, the application is a continuation of Application 06/889,722, filed July 28, 1986 (ABN), which is a Division of 06/291,892, filed August 11, 1981 (ABN), which is a continuation-in-part of 06/190,799, filed September 25, 1980 (ABN).

Claim 25 is representative:

25. A composition comprising water and a nonglycosylated polypeptide having the amino acid sequence of a mature human fibroblast interferon, said nonglycosylated polypeptide having a total of 165 or 166 amino acids and said composition being free of any glycosylated human fibroblast interferon.

The references relied on by the examiner are:

Havell et al. (Havell), The Journal of Biological Chemistry, "Altered Molecular Species of Human Interferon Produced in the Presence of Inhibitors of Glycosylation", Vol. 252, No. 12, pages 4425-4427 (1977).

Goeddel et al. (Goeddel), Nature, "Direct expression in *Escherichia coli* of a DNA sequence coding for human growth hormone", Vol. 281, pages 544-548 (1979).

Taniguchi et al. (Taniguchi), Gene, "The nucleotide sequence of human fibroblast interferon cDNA", Vol. 10, pages 11-15 (1980).

A newly cited reference, relied on by this Board, is:

Darnell et al. (Darnell), Molecular Cell Biology, "Golgi Vesicles: Sorting and Glycosylation of Secretory and Membrane Proteins", Second Edition, pages 661-667 (1990).

The issues presented for review are:

(1) Whether the examiner correctly rejected claims 25, 28-30, 32, and 33 under 35 USC 103 as unpatentable over Havell; and

(2) Whether the examiner correctly rejected claims 25 and 28-30 under 35 USC 103 as unpatentable over Taniguchi in view of Goeddel.

For the reasons discussed infra, we do not sustain either of those rejections.

DELIBERATIONS

Our deliberations in this matter have included evaluation and review of the following materials:

(1) The instant specification, including Figures 1-6, and all of the claims on appeal;

(2) Appellants' main Brief before the Board, and the declaration evidence referred to in the main Brief;

(3) The examiner's Answer;

(4) The prior art references cited and relied on by the examiner;

(5) The above-cited Darnell textbook, pages 661-667; and

(6) The opinion and decision of the previous Board panel in parent application Serial No. 06/291,892 (Appeal No. 609-97 decided December 30, 1985, adhered to on reconsideration March 28, 1986).

This record does not reflect that the examiner entered appellants' Reply Brief and, accordingly, we have not considered appellants' Reply Brief before the Board.

FINDINGS

(1) In parent application Serial No. 06/291,892, the previous Board panel affirmed the examiner's decision refusing to allow claims 1, 2 and 4 (Appeal No. 609-97 decided December 30, 1985, decision adhered to on reconsideration March 28, 1986). This appeal involves different claims, different arguments, and a substantially different record compared with the previous appeal. Accordingly, we have considered the matter anew;

(2) This appeal and Appeal No. 93-1250 involve closely related subject matter and common issues. For that reason, appellants filed a "Request to Consolidate Oral Hearings", see Paper No. 36 in the file. That request was granted "by Order of the Board" in Paper No. 37, and a consolidated Oral Hearing was held August 9, 1994, involving both appeals;

(3) In proceedings before the PTO, claims in an application are to be given their broadest reasonable interpretation consistent with the specification, and claim language should be read in light of the specification as it would be interpreted by one of ordinary skill in the art. In re Sneed, 710 F.2d 1544, 218 USPQ 385 (Fed. Cir. 1983);

(4) Following that principle of claim interpretation, we find that the recitation "mature human fibroblast interferon" in independent claim 25 connotes the bacterial or other microbial production of an interferon molecule unaccompanied by associated glycosylation and the presequence that immediately attends mRNA translation of the human fibroblast interferon genome. See the instant specification, page 5;

(5) As stated by appellants in the specification, page 4,

> We perceived that application of recombinant DNA technology would be the most effective way of providing large quantities of fibroblast interferon which, despite the absence in material so produced of the glycosylation characteristic of human-derived material, could be employed clinically in the treatment of a wide range of viral and neoplastic diseases [emphasis added];

(6) As stated in appellants' main Brief before the Board, page 35, naturally-occurring human fibroblast interferon is glycosylated. This is consistent with appellants' reference in the specification, page 4, to the "glycosylation characteristic of human-derived material" and also with the examiner's finding in the Answer, page 10, that "fibroblast interferon is known to be glycosylated";

(7) Independent claim 25 recites "a nonglycosylated polypeptide" and further contains the limitation "said composition being free of any glycosylated human fibroblast interferon". In view

of those claim recitations, we find that appellants' mature human fibroblast interferon, in claim 25, is completly nonglycosylated;

(8) In rejecting claims 25, 28-30, 32, and 33 under 35 USC 103 as unpatentable over Havell, the examiner argues that it would have been obvious to modify the subject matter disclosed by Havell in such manner to arrive at the nonglycosylated polypeptide defined in the appealed claims. Likewise, in rejecting claims 25 and 28-30 under 35 USC 103 as unpatentable over Taniguchi in view of Goeddel, the examiner argues that a person having ordinary skill would have been motivated to produce appellants' nonglycosylated polypeptide using recombinant expression in E. coli "because of the known benefits of such a procedure (greater yields, faster production, ability to obtain high purity)". See the examiner's Answer, page 4. We disagree;

(9) With respect to each prior art rejection, the examiner's position presupposes that the hypothetical person having ordinary skill in this art would have reasonably expected appellants' nonglycosylated polypeptide to possess biological activity. This is not the case;

(10) As stated by appellants in their main Brief before the Board, page 35, and not controverted by the examiner,

> [N]aturally-occurring human fibroblast interferon is glycosylated, and one skilled in the art in 1980 would not have been able to predict whether...nonglycosylated human fibroblast interferon, if produced, would be

> biologically active. Appellants emphasize that the examiner has cited no evidence... that would have led one skilled in the art to conclude that completely nonglycosylated human fibroblast interferon would be biologically active;

(11) According to teachings in the above-cited Darnell textbook, Molecular Cell Biology, page 667, the conformation and stability of proteins may well be affected by N-linked and O-linked oligosaccharides. Respecting the impact of glycosylation or absence of glycosylation on proteins in general, Darnell states that

> Because the impact of the absence of glycosylation is so variable and by no means absolute, researchers have concluded that sugar residues play no mandatory role in the movement of proteins to the cell surface: they are not a "ticket" needed to move through the transport organelles. In all likelihood, carbohydrates play a role in ensuring the correct charge, conformation, and stability of maturing proteins. For some proteins, this function is apparently superfluous; for others, it is clearly necessary;

(12) We find that Darnell's teachings, reproduced in Finding (11), reinforce the position in the main Brief, page 35, that "one skilled in the art in 1980 would not have been able to predict whether...nonglycosylated human fibroblast interferon, if produced, would be biologically active". Again, that position is not controverted by the examiner.

CONCLUSIONS

(1) On the particular facts of this case, we hold that a person having ordinary skill in the art would not have reasonably expected appellants' nonglycosylated polypeptide to possess biological activity. In so holding, we have attempted to place ourselves "back in time" when the invention was made. Cf. Ex parte Aggarwal, 23 USPQ2d 1334 (BPAI 1992) (§103 rejection of claims drawn to nonglycosylated protein reversed where record does not establish that the nonglycosylated material reasonably expected to have biological activity);

(2) On the particular facts of this case, we hold that a person having ordinary skill would not have a sufficient basis for the requisite, reasonable expectation of success to sustain a rejection of claims 25, 28-30, 32, and 33 under 35 USC 103. This holding is dispositive with respect to each §103 rejection set forth by the examiner;

(3) Accordingly, we do not sustain the rejection of claims 25, 28-30, 32, and 33 under 35 USC 103 as unpatentable over Havell. Nor do we sustain the rejection of claims 25 and 28-30 under 35 USC 103 as unpatentable over Taniguchi in view of Goeddel;

(4) The examiner's decision, refusing to allow claims 25, 28-30, 32, and 33, is reversed.

REVERSED

SHERMAN D. WINTERS)
Administrative Patent Judge)
)
)
)
WILLIAM F. SMITH) BOARD OF PATENT
Administrative Patent Judge) APPEALS
) AND
) INTERFERENCES
)
TEDDY S. GRON)
Administrative Patent Judge)

Appeal No. 94-2099
Application 07/365,284

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